P1 1184822

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office

June 22, 2004

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE.

APPLICATION NUMBER: ***60/456,882***
FILING DATE: ***March 21, 2003***
RELATED PCT APPLICATION NUMBER: *PCT/US04/08739*

REC'D 28 JUN 2004	
WIPO	PCT

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

N. WOODSON
Certifying Officer

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

03-24-03

Approved

Please type a plus sign (+) inside this box → ⊞

PTO/SB/16 (8-00)
Approved for use through10/31/2002. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Kenneth A.	Murdock	6445 East Right Hand Fork, Hobblecreek Canyon Springville, Utah USA
Alexander G.	Schauss	2722 Pioneer Way East, Tacoma, Washington, USA

☐ *Additional inventors are being named on the ___ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (280 characters max)

Jucari and Acai Fruit Based Dietary Supplements

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

☐ Customer Number [ ] → *Place Customer Number Bar Code Label here*

OR *Type Customer Number here*

☒ Firm or Individual Name	Brent T. Winder				
Address	Jones, Waldo, Holbrook & McDonough				
Address	170 South Main Street, Suite 1500				
City	Salt Lake City	State	Utah	ZIP	84101
Country	USA	Telephone	801 521-3200	Fax	801 328-0537

ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages* 6 ☐ CD(s), Number [ ]

☐ Drawing(s) *Number of Sheets* [ ] ☒ Other (specify) Articles

☒ Application Data Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☒ Applicant claims small entity status. See 37 CFR 1.27.

☐ A check or money order is enclosed to cover the filing fees.

☒ The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 50-1723

☐ Payment by credit card. Form PTO-2038 is attached.

FILING FEE AMOUNT ($) $80.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☐ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE Brent T. Winder Date 3/21/03

TYPED or PRINTED NAME Brent T. Winder REGISTRATION NO. *(if appropriate)* 46,250

TELEPHONE 801 521-3200 Docket Number: 10107.0011

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. The information is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington, D.C. 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, D.C. 20231.

[redacted]

60456882 .032103

PTO/SB/17 (09-00)
Approved for use through 10/31/2002. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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FEE TRANSMITTAL for FY 2001

Patent fees are subject to annual revision.

TOTAL AMOUNT OF PAYMENT ($) 80

Complete if Known	
Application Number	
Filing Date	
First Named Inventor	Kenneth A. Murdock
Examiner Name	
Group Art Unit	
Attorney Docket No.	10107.0011

METHOD OF PAYMENT

1. [X] The Commissioner is hereby authorized to charge indicated fees and credit any overpayments to:

Deposit Account Number: 50-1723

Deposit Account Name: Jones, Waldo, Holbrook & McDonough

[X] Charge Any Additional Fee Required Under 37 CFR 1.16 and 1.17

[X] Applicant claims small entity status. See 37 CFR 1.27

2. [ ] Payment Enclosed:

[ ] Check [ ] Credit card [ ] Money Order [ ] Other

FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
101	710	201	355	Utility filing fee	
106	320	206	160	Design filing fee	
107	490	207	245	Plant filing fee	
108	710	208	355	Reissue filing fee	
114	150	214	75	Provisional filing fee	80

SUBTOTAL (1) ($) 80

2. EXTRA CLAIM FEES

			Extra Claims		Fee from below		Fee Paid
Total Claims		-20** =		X		=	0
Independent Claims		- 3** =		X		=	0
Multiple Dependent						=	0

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
103	18	203	9	Claims in excess of 20
102	80	202	40	Independent claims in excess of 3
104	270	204	135	Multiple dependent claim, if not paid
109	80	209	40	** Reissue independent claims over original patent
110	18	210	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($) 0

**or number previously paid, if greater; For Reissues, see above

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
105	130	205	65	Surcharge - late filing fee or oath	
127	50	227	25	Surcharge - late provisional filing fee or cover sheet	
139	130	139	130	Non-English specification	
147	2,520	147	2,520	For filing a request for *ex parte* reexamination	
112	920*	112	920*	Requesting publication of SIR prior to Examiner action	
113	1,840*	113	1,840*	Requesting publication of SIR after Examiner action	
115	110	215	55	Extension for reply within first month	
116	390	216	195	Extension for reply within second month	
117	890	217	445	Extension for reply within third month	
118	1,390	218	695	Extension for reply within fourth month	
128	1,890	228	945	Extension for reply within fifth month	
119	310	219	155	Notice of Appeal	
120	310	220	155	Filing a brief in support of an appeal	
121	270	221	135	Request for oral hearing	
138	1,510	138	1,510	Petition to institute a public use proceeding	
140	110	240	55	Petition to revive - unavoidable	
141	1,240	241	620	Petition to revive - unintentional	
142	1,240	242	620	Utility issue fee (or reissue)	
143	440	243	220	Design issue fee	
144	600	244	300	Plant issue fee	
122	130	122	130	Petitions to the Commissioner	
123	50	123	50	Petitions related to provisional applications	
126	240	126	240	Submission of Information Disclosure Stmt	
581	40	581	40	Recording each patent assignment per property (times number of properties)	
146	710	246	355	Filing a submission after final rejection (37 CFR § 1.129(a))	
149	710	249	355	For each additional invention to be examined (37 CFR § 1.129(b))	
179	710	279	355	Request for Continued Examination (RCE)	
169	900	169	900	Request for expedited examination of a design application	

Other fee (specify) ____________

*Reduced by Basic Filing Fee Paid

SUBTOTAL (3) ($) 0

SUBMITTED BY				Complete *(if applicable)*	
Name *(Print/Type)*	Brent T. Winder	Registration No. *(Attorney/Agent)*	46,250	Telephone	801 521-3200
Signature	[signature]			Date	3/21/03

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

Burden Hour Statement: This form is estimated to take 0.2 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Washington, DC 20231.

[redacted]

JONES WALDO HOLBROOK & MCDONOUGH PC

PATENT APPLICATION
Docket No. 10107.0011
EXPRESS MAIL LABEL: EV 057 526 791 US
ATTORNEYS & COUNSELORS
EST. 1875

TEL: 801-521-3200
FAX: 801-328-0537

170 SOUTH MAIN ST, SUITE 1500
SALT LAKE CITY, UTAH 84101

WWW.JONESWALDO.COM

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

BOX PROVISIONAL PATENT APPLICATION
Assistant Commissioner for Patents
Washington, DC 20231

Sir/Madam:

Transmitted herewith for filing is the provisional patent application of Kenneth A. Murdock and Alexander G. Schauss for **JUCARI AND ACAI FRUIT BASED DIETARY SUPPLEMENTS** comprising 6 pages of specification and claims.

Enclosed also are:

X Various Articles

X Provisional Application for Patent Cover Sheet

X Fee Transmittal for FY 2001

X A Certificate of Mailing by "Express Mail" certifying a filing date of March 21, 2003, by use of Express Mail Label No. EV 057 526 791 US.

X Return Postcard

CERTIFICATE OF DEPOSIT
EXPRESS MAIL LABEL NO.: EV 057 526 791 US DATE OF DEPOSIT: March 21, 2003

I hereby certify that this paper or fee is being deposited with the United States Postal Service Express Mail Post Office to Addressee service under 37 C.F.R. § 1.10 on the date indicated above and is addressed to: Box Patent Application, Assistant Commissioner for Patents, Washington, D.C. 20231

Jan S. Mahoney, Legal Assistant to
Brent P. Winder

584902v1

The filing fee has been calculated as shown below.

			SMALL ENTITY		OTHER THAN A SMALL ENTITY	
FOR	NO. FILED	NO. EXTRA	RATE	FEE	RATE	FEE
BASIC FEE				$80		$160
TOT. CLAIMS	-20 =		X 9 =	$	X 18 =	$
IND. CLAIMS	-30 =		X 39 =	$	X 78 =	$
MULTIPLE DEPENDENT CLAIMS PRESENTED						
ASSIGNMENT FILING FEE			$40		$40	
			TOTAL	$80	TOTAL	

X The Commissioner is hereby authorized to charge payment of the following fees associated with this communication or credit any overpayment to Deposit Account No. 50-1723, and any additional filing fees required under 37 C.F.R. § 1.16.

Please address all future correspondence in connection with the above-identified patent application to the attention of the undersigned.

Dated this 21st day of March, 2003.

Respectfully submitted,

Brent T. Winder
Registration No. 46,250
Attorneys for Applicant

JONES, WALDO, HOLBROOK & MCDONOUGH, PC
170 South Main Street, Suite 1500
Salt Lake City, Utah 84101-1644
Telephone: (801) 521-3200

BTW/jsm
Docket: 10107.0011
Enclosures

CERTIFICATE OF MAILING BY EXPRESS MAIL

"Express Mail" Mailing Label No. * EV 057 526 791 US

Date of Deposit: * March 21, 2003

I hereby certify that the enclosed new patent application entitled "**JUCARI AND ACAI FRUIT BASED DIETARY SUPPLEMENTS,**" along with the accompanying documents – Various articles, a return postcard, Fee Transmittal for FY 2001; Provisional Patent Application Cover Sheet, and a transmittal letter to the Patent Office, in the name of Kenneth A. Murdock and Alexander G. Schauss – is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. Section 1.10 on the date indicated above in an envelope addressed to the Assistant Commissioner of Patents, Box Patent Applications, Washington, D.C. 20231.

Respectfully submitted this 21 day of March, 2003.

Respectfully submitted,

JONES, WALDO, HOLBROOK & McDONOUGH

Legal Assistant to
Brent T. Winder
Registration No. 46,250
Attorney for Applicant
170 South Main Street, Suite 1500
Salt Lake City, UT 84101-1644
Telephone: (801) 521-3200

Attorney Docket No. 10107.0011

584907v1

[redacted]

United States Patent Application

for

JUCARI AND ACAI FRUIT BASED DIETARY SUPPLEMENTS

TO THE COMMISSIONER OF PATENTS AND TRADEMARKS:

Kenneth A. Murdock, a citizen of the United States, who resides at **6445 East Right Hand Fork, Hobblecreek Canyon, Springville, Utah 84663**, and whose post office address is **P.O. Box 655, Springville, Utah 84663-0655**, and **Alexander G. Schauss**, a citizen of the United States, who resides at **2722 Pioneer Way East, Tacoma, Washington 98404**, and whose post office address is **P.O. Box 1174, Tacoma, Washington 98401**, pray that letters patent be granted to them as inventors of **JUCARI AND ACAI FRUIT BASED DIETARY SUPPLEMENTS** as set forth in the following specification.

Background

A well-known species of palm is the Acai, or Cabbage Palm (Euterpe oleracea), which is a palm tree characteristic of the northern region of Brazil known as Para. The Acai is characterized by a thin trunk and round egg-shaped clustered fruits that are dark purple, sometimes even verging on black when ripe. The pulp of the Acai fruit is also a deep, dark purple.

The oil from the fruit mesocarp is known to contain a high proportion of mono-unsaturated and polyunsaturated fatty acids, and a relatively low concentration of saturated fat and trans fatty acids. The fruit is also known to be rich in lipids, fibers and protein. Studies further indicate that the fruit contains substantial levels of alpha-tocopherols (Vitamin E) and anthocyanins. Anthocyanins are known antioxidants—which scavenge free radicals (which free radicals have been linked to many chronic degenerative diseases, including heart disease, arthritis and cancer).

Prior studies have wavered significantly on the amount of anthocyanins that are present in the Acai fruit. For example, one study indicated that the amount of anthocyanins in the Acai fruit is on the order of 336 mg/100 g of fruit. Other studies have concluded that the concentrations can be as high as 1347 mg/ 100 g fruit. Thus, it is clear that the methodology for determining anthocyanin concentrations has historically been unreliable. This has led, in part, to an under-utilization of the Acai fruit, and all the nutrients therein.

Another factor that has contributed to the under-utilization of the Acai fruit is that it is very prone to deterioration. Specifically, due to microbial contamination by bacteria, fungi and yeast, the fruit and juice of the Acai fruit deteriorate rapidly. It is believed that

the microbial contamination originates from the trees, the fruits rubbing together after picking, and from the soil (fecal coliforms). The level of deterioration also depends on the amount of time that transpires between picking the fruits and marketing them. It is also noted that almost half of the anthocyanins degrade two days after the fruit is picked.

Some methods that have been utilized to improve shelf life of the Acai fruit include gaseous sulfiting and blanching. These methods improve the sanitary quality and help preserve the anthocyanins. Pasteurization of the drink is also employed whereby peroxidases and polifenoloxidases are inactivated and pathogens, fungi and yeasts are destroyed. The addition of citric acid also facilitates the treatment and partially protects the anthocyanins.

In an effort to overcome the rapid deterioration of Acai fruit and juice, and thereby expose the product to broader markets, some companies have tried freezing the fruit pulp. However, simply freezing the Acai fruit pulp in this manner requires careful monitoring of the temperature—with even relatively slight deviations in temperature resulting in the activation of deteriorating enzymes and fermenting agents. Moreover, when thawing such frozen fruit pulp for use, these agents also become activated resulting in grittiness to the pulp.

The foregoing problems, among others, have been resolved by the present invention. Specifically, it has recently been discovered through ORAC analysis that antioxidant activity in Acai fruit is much higher than was heretofore known.

Because of this discovery, it has now become apparent that Acai fruit is a good candidate for developing a dietary supplement. In other words, prior to this discovery of the extremely high antioxidant concentrations in the Acai fruit, the fruit was used

primarily as an energy drink or as part of a frozen treat that also had some antioxidant properties as well as some protein, vitamin and mineral content. However, through use of the ORAC methodology, it has become apparent that Acai fruit juice and pulp could be further processed into a powder that could then be used as a dietary supplement that could be combined with any number of foods.

It is also noted that by preparing the Acai fruit into a powder in this manner, the concerns of fruit preservation mentioned above are addressed. Specifically, the present process allows the highly nutritious features of the fruit to be preserved and enjoyed, without the associated concerns of rapid microbial degradation. As is apparent in the attached commissioned study, it has been discovered that when the pulp is freeze-dried the microbial activity virtually ceases.

While the foregoing discussion focuses primarily on Acai fruits and juices, Applicant has also discovered through the ORAC methodology that the Jucara palm (Euterpa edulis) fruit also has extremely high antioxidant activity.

For example, it has been discovered that Jucara fruit contains a very high level of proanthocyanidins. It was also discovered that the Jucara fruit exhibited high antioxidant activities against hydroxy radical and peroxynitrite.

Description of one Embodiment of the Fruit Harvesting/Supplement Manufacturing Process

When ripe, the Acai and Jucara fruit is harvested and processed. In one method of processing, the fruit pit and fibrous materials are removed with a centrifuge pulping machine. The end product is a pulp of Acai and the waste product being the nut-like center of the fruit with some attached fibrous material. The pulp is then packaged sometimes in a vertical form fill and seal machine or other packaging. The packaged pulp can then be immediately frozen and shipped in frozen form to the end user.
In one embodiment of the present invention, the frozen pulp (or before freezing, depending upon location), is freeze dried (vacuum process) which causes concentration of the active ingredients. The end product is ground into a power and then packaged. Once ground into a powder, the pulp-based supplement can be combined with numerous food products including, but not limited to ice cream, yogurt, and dairy products (including cheese), beverage coloring agents, food coloring agents, sports bars, protein powders, tapioca, and a wide range of other nutraceutical applications. The powder supplement can also be added as a garnish to sauces and soups.

Claims

What is claimed is:

1) A powdered dietary supplement comprising dehydrated Acai fruit extract.

2) A method of preparing a dietary supplement comprising the steps of:

   a) providing an Acai fruit extract;

   b) drying the extract to form a powder, wherein the powder is the dietary supplement.

3) A powdered dietary supplement comprising dehydrated Jucara fruit extract.

4) A method of preparing a dietary supplement comprising the steps of:

   c) providing an Jucara fruit extract;

   d) drying the extract to form a powder, wherein the powder is the dietary supplement.

PROVISIONAL PATENT APPLICATION

OF

KENNETH A. MURDOCK AND ALEXANDER G. SCHAUSS

FOR

JUCARI AND ACAI FRUIT BASED DIETARY SUPPLEMENTS

Attorney Docket No. 10107.0011

[redacted]

SILLIKER, Inc.
Illinois Laboratory
1304 Halsted Street
Chicago Heights, IL 60411
708/ 756 3210 Fax 708/ 756 0049

CERTIFICATE OF ANALYSIS

COA No:	CHG-17193280-0
Supersedes:	None
COA Date	3/14/03
Page 1 of 1	

TO:
Ms. Tracy Winter
Silliker Laboratory Research
160 Armory Drive
South Holland, IL 60473

Received From:	South Holland, IL
Received Date:	2/27/03

Location of Test: (except where noted)
Chicago Heights, IL

Analytical Results

Desc. 1: AIBMR Inc.
Desc. 2: Jucara Freeze Fried Fruit
Desc. 3: Powder

Laboratory ID: 171378575
Condition Rec'd: NORMAL
Temp Rec'd (°C): 16

Analyte	Result	Units	Method Reference	Test Date	Loc.
Amino Acids Complete			USDA MSS2 (1993)	3/14/03	
Sample Wt, Cystine	0.0326	g			
Aspartic Acid	0.12	%			
Threonine	0.04	%			
Serine	0.05	%			
Glutamic Acid	0.10	%			
Glycine	0.04	%			
Alanine	0.05	%			
Valine	0.05	%			
Methionine	0.02	%			
Isoleucine	0.03	%			
Leucine	0.06	%			
Tyrosine	0.02	%			
Phenylalanine	0.04	%			
Lysine	0.05	%			
Histidine	0.02	%			
Arginine	0.04	%			
Proline	0.05	%			
Hydroxyproline	<0.01	%			
Cystine	0.03	%			
Tryptophan	0.06	%			

Randy Fleener — Laboratory Director

SILLIKER, Inc.
Illinois Laboratory
1304 Halsted Street
Chicago Heights, IL 60411
708/ 756 3210 Fax 708/ 756 0049

CERTIFICATE OF ANALYSIS

COA No:	CHG-17193246-0
Supersedes:	None
COA Date	3/14/03
Page 1 of 2	

TO:
Ms. Tracy Winter
Silliker Laboratory Research
160 Armory Drive
South Holland, IL 60473

Received From:	South Holland, IL
Received Date:	2/27/03

Location of Test: (except where noted)
Chicago Heights, IL

Analytical Results

Desc. 1: AIBMR Inc.
Desc. 2: Jucara Freeze Fried Fruit
Desc. 3: Powder

Laboratory ID: 171378581
Condition Rec'd: NORMAL
Temp Rec'd (°C): 16

Analyte — Nutrition - Mandatory
Test Date 3/14/03 **Loc.**

NUTRITIONAL ANALYSIS

Serving Size: 100 grams

LABEL ANALYTES		ANALYTICAL DATA PER 100g	ANALYTICAL DATA PER SERVING	ROUNDED DATA PER SERVING	% DAILY VALUE
Calories		370.2	370.2	370	
Calories from Fat		22.4	22.4	20	
Total Fat	(g)	2.48	2.48	2.5	4
Saturated Fat	(g)	0.68	0.68	0.5	2
Cholesterol	(mg)	<1.0	<1.0	0	0
Sodium	(mg)	25.5	25.5	25	1
Total Carbohydrate	(g)	86.3	86.3	86	29
Dietary Fiber	(g)	0.83	0.83	<1	4
Sugars	(g)	<0.10	<0.10	0	
Protein (F=6.25)	(g)	0.68	0.68	<1	
Vitamin A	(IU)	179	179		4
Vitamin C	(mg)	<1.0	<1.0		*
Calcium	(mg)	33.0	33.0		4
Iron	(mg)	0.53	0.53		2
CONTRIBUTING ANALYTES					
Moisture	(g)	8.62	8.62		
Ash	(g)	1.93	1.93		
Beta Carotene	(IU)	179	179		
Retinol	(IU)	<5	<5		
Vit A % Beta Carotene		100			

* Contains less than 2% of the Daily Value of this nutrient.

SUGAR PROFILE

Dextrose	<0.10	(g/100g)	Fructose	<0.10	(g/100g)
Lactose	<0.10	(g/100g)	Maltose	<0.10	(g/100g)
Sucrose	<0.10	(g/100g)			

[redacted]

SILLIKER, Inc.
Illinois Laboratory
1304 Halsted Street
Chicago Heights, IL 60411
708/ 756 3210 Fax 708/ 756 0049

CERTIFICATE OF ANALYSIS

COA No:	CHG-17193246-0
Supersedes:	None
COA Date	3/14/03
Page 2 of 2	

TO:
Ms. Tracy Winter
Silliker Laboratory Research
160 Armory Drive
South Holland, IL 60473

Received From:	South Holland, IL
Received Date:	2/27/03

Location of Test: (except where noted)
Chicago Heights, IL

Analytical Results

SATURATED FATTY ACID	FORMULA	%
Butyric	4:0	<0.1
Caproic	6:0	<0.1
Caprylic	8:0	<0.1
Capric	10:0	<0.1
Undecanoic	11:0	<0.1
Lauric	12:0	<0.1
Tridecanoic	13:0	<0.1
Myristic	14:0	0.1
Pentadecanoic	15:0	<0.1

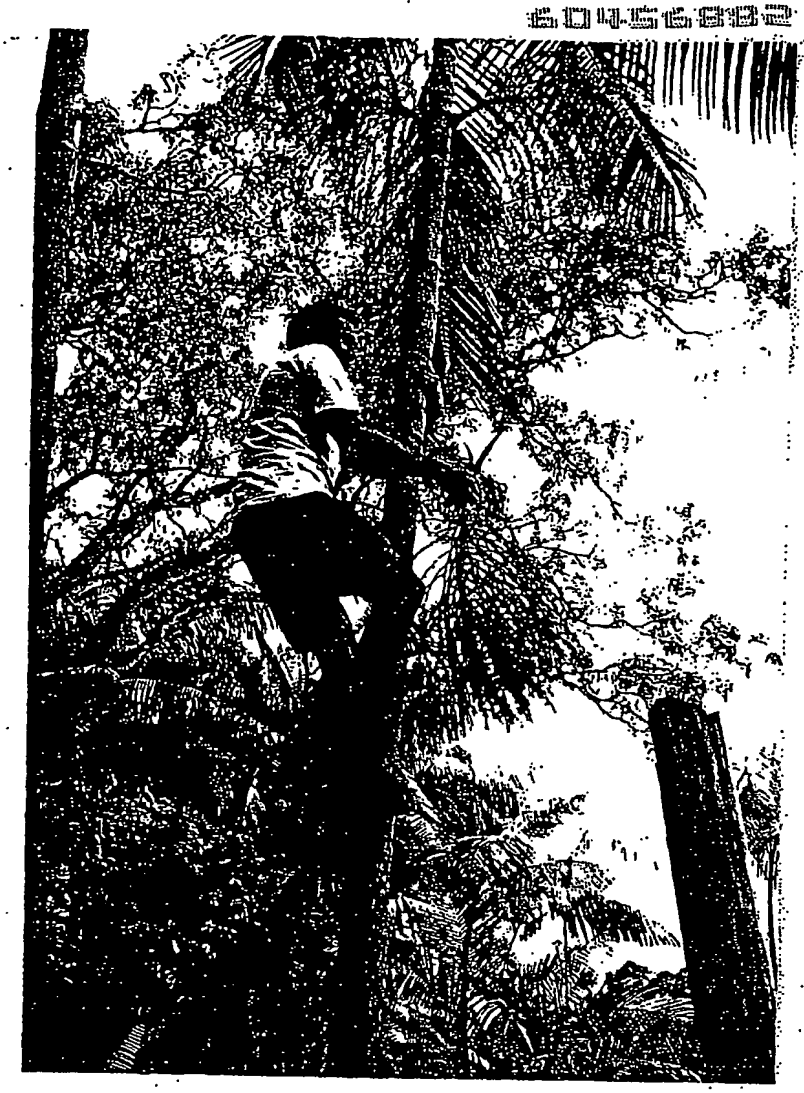
SATURATED FATTY ACID	FORMULA	%
Palmitic	16:0	26.7
Margaric	17:0	<0.1
Stearic	18:0	1.7
Nonadecanoic	19:0	<0.1
Eicosanoic	20:0	0.2
Behenic	22:0	<0.1
Tricosanoic	23:0	<0.1
Lignoceric	24:0	<0.1

MONOUNSATURATED FATTY ACID	FORMULA	%
Tridecenoic	13:1	<0.1
Myristoleic	14:1	<0.1
Pentadecenoic	15:1	<0.1
Palmitoleic	16:1	6.3
Margaroleic	17:1	<0.1
Oleic	18:1C	53.9
Elaidic	18:1T	<0.1
Gadoleic	20:1	<0.1
Erucic	22:1	<0.1
Nervonic	24:1	<0.1

POLYUNSATURATED FATTY ACID	FORMULA	%
Linoleic	18:2	10.0
Linolenic	18:3	1.1
Gamma Linolenic	18:3G	<0.1
Eicosadienoic	20:2	<0.1
Eicosatrienoic	20:3	<0.1
Homogamma Linolenic	20:3G	<0.1
Arachidonic	20:4	<0.1
Eicosapentaenoic	20:5	<0.1
Docosadienoic	22:2	<0.1
Docosahexaenoic	22:6	<0.1

Total Polyunsaturated Fatty Acid	11.10
Total Monounsaturated Fatty Acid	60.20
Total Saturated Fatty Acid	28.70

Randy Fleener — Laboratory Director

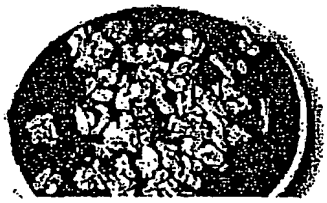


Collecting Acai

[redacted]

the sender.

****



Reply Reply All Forward Delete Previous Next Close

[redacted]

Preparing, washing Acai before depulping.

Depulping/packaging of Acai pulp

Acai (*Euterpe oleracea*, Cabbage Palm) Summary of Scientific Information

Prepared by: Diane Verrilli
2/5/01; Updated 3/6/01
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Summary of Available Information (only referenced material was included):

Background: Acai (*Euterpe oleracea*, Martius) of the family Palmaceae, is a palm tree characteristic of the northern region of Brazil, known as Para (laderoza et al, 1992). *Euterpe oleracea* (or Cabbage Palm) occurs in the Amazon estuary, on lands of the flood plains, the back swamps and on the uplands. The palm has a thin trunk, which is sometimes slightly curved (Cavalcante s translation, 1977). The fruits are round or egg-like in shape, and are clustered into bunches. The external color is dark purple, almost black when mature, and the pulp is the same color (laderoza et al., 1992). Fruiting occurs through out the year, with the dry season, July to December, being the period of greatest abundance (Cavalcante, 1977).

Synonyms: Açai is known by a number of other names, for instance, in Brazil it is known as Acal, Acaizeiro, Assai, Jicara, Jucara, Palmiteiro, Piria. In Colombia, it is known as Assai and Manaca, whereas the Suriname names are Manaka, Pinapalm, Prasara, Wapoe, and Wasei (Plotkin & Balick, 1984).

Medicinal Uses: The Matowai Bush people of Surimane apply the sap to cuts as a homeostatic. A homeostatic agent refers to homeostatis, or the state of equilibrium or balance in the body. The fruit furnishes a dark green oil used in rural medicine, principally as an anti-diarrheal agent.

Other Uses: The palmito (also called hearts-of-palm or palm-hearts) is edible and is exported and used regionally, adding income to the economy. The oil from the fruit pulp has industrial applications. The

fruit is used to make ice cream and a beverage in Peru and Brazil. This species might also be the source of a strong structural fiber (Plotkin & Balick, 1984).

Economic Value in Brazil: Acai is highly valuable in the floodplains near Belem, Brazil. The data collected in 1996 presented values for managed secondary forests ranging from US$ 896 to 1814 at a 30% interest rate. This data shows why acai is highly valuable on these lands, and also explains why so may hectares have been converted to intense acai managed forests in this region. Presently, there is a large urban market for acai, which has developed over the last 25 years. Acai production shows seasonal patterns, and therefore, it is important to take seasonal variation into account when estimating revenue (Miret et al, 1996).

The Juice: The juice is prepared from the acai fruit, by either manual or mechanical processes. The juice, simply referred to as acai, is a main component in the diet of the lower classes. It is consumed in a number of ways, such as with manioc meal or with tapioca and sugar, with manioc meal and grilled fish or dried shrimp, as a porridge (*mingau*) made with manioc meal, and as an ice cream or pop-sickle flavoring (Cavalcante, 1977). The juice from the acai fruit is used as the main ingredient in a popular beverage in Brazil and Peru (Plotkin & Balick, 1984; Greater Continents do Brasil, marketing information, 2000).

Nutritive Value: The following was stated by Chaves and Pechnik, 1945, in reference to acai s nutritive value: the interpretation of analytic data permits us to ascertain it to be an essentially energetic food, with a caloric value higher than that of milk and which a content of lipoids (lipase per Biochemical and Technical Studies summary from GCB) is twice as high as that of milk. It is not very rich in proteins, and the percentage of glycosides is not very high. Nevertheless, acai, as it is commonly consumed, with sugar and starch, may be considered a rich food of high caloric value. The content of minerals, calcium, phosphorus, and iron reveals benefits (Chaves & Pechnick, 1945:6, reference is not obtainable to date; information is from Cavalcante, 1977). Another researcher conducted biological experiments with rats, and showed a presence of Vitamin A

in the fruit (Dante Costa, 1959:51, reference is not obtainable to date; information is from Cavalcante, 1977). The juice (drink) is slightly acidic (pH = 5.2) and rich in lipids, fibers, and proteins (52, 25, and 10% of the dry matter, respectively). High levels of alpha-tocopherols (45mg/100 grams), commonly known as vitamin E, and anthocyanins (1 gram/100grams) make it an excellent source of antioxidants. Smaller fruits have been shown to have higher levels of anthocyanins. Trace elements present are manganese, copper, boron, and chromium (Rogez, 2000:IX — X).

Nutrient Composition Table: Acai Pulp (Data taken from table 22, Rogez, 2000:158 & Greater Continents do Brasil technical information, 2000)

Component	Tavares & Costa (1936)	Chaves & Pech-nick (1948)	INCAP (1961)	Almeida & Valsechi (1966)	ENDEF (1977)
pH	-	-	-	5.9	-
Energy (Kcal per 100 grams)	111.7	80	265	-	247
Dry Material (%)	61.4	59.00	59.0	49.1	54.1
Protein (1)	5.70	5.73	5.76	9.6	7.02
Lipids (total) (1)	17.92	22.71	20.68	24.8	22.55
Sugars (total) (1)	0.65	20.37	20.68	24.5	36.41
Reducer (total) (1)	-	-	-	19.4	-
Fructose (1)	-	-	-	-	-
Glucose	-	-	-	-	-

(1) Sucrose	-	-	-	4.8	-
(1) Fiber (cellulose)	74.59	30.51	30.51	32.4	31.24
(1) Ash	1.14	2.12	2.03	3.6	2.77
(1) Sodium (micro)	-	-	-	69	-
(2) Potasium (macro)	-	-	-	1185	-
(2) Calcium (macro)	-	-	-	241	218
(2) Magnesium (macro)	-	-	-	140	-
(2) Iron (micro)	-	-	-	24	21.8
(2) Copper (micro)	-	-	-	-	-
(2) Zinc (micro)	-	-	-	-	-
((2) Sulfur	-	-	-	112	-
(2) Phos-phorus (macro)	-	-	-	119	107
(2) Vitamin C	-	-	-	-	17
(2) Vitamin	-	-	-	-	0.67

B1 (thiamin) (2)					
Vitamin B2 (ribo-flavin) (2)	-	-	-	-	0.02
Vitamin B3 (niacin) (2)	-	-	-	-	0.7
Alpha-tocoperol (Vit E) (2)	-	-	-	-	-

(1) g/100g = grams/100 grams dry material
(2) mg/100g = milligrams/100 grams dry material
Macro = ? (Came from translated chart)
Micro = ? (Came from translated chart)
Taken from: Rogez, 2000, Table 22, p.158; Translations from Greater Continents do Brasil, Ltda.

Nutrient Composition of Acai Juice:

Compo-nent	Motta (1946)	Chaves & Pech-nick (1948)	Lehti (1993)	Rogez et al. (1996)
pH	-	-	-	5.8
Energy (Kcal per 100 grams)	-	-	-	66.3
Dry Material (%)	13.0	15.0	15	15

Protein (1)	18.23	8.33	-	13
Lipids (total) (1)	45.85	50.67	-	48
Sugars (total) (1)	-	6.67	-	1.5
Reducer (total) (1)	-	-	-	1.5
Fructose (1)	-	-	-	0
Glucose (1)	-	-	-	1.5
Sucrose (1)	-	-	-	0
Fiber (cellulose) (1)	32.30	32.33	-	34.0*
Ash (1()	3.62	2.00	-	3.5
Sodium (micro) (2)	-	-	16	56.4
Potassium (macro) (2)	-	-	499	932
Calcium (macro) (2)	275	133	-	286
Magnesium (macro) (2)	-	-	121	174
Iron (micro) (2)	5	10.2	26	1.5

Copper (micro) (2)	-	-	2	1.7
Zinc (micro) (2)	-	-	2	7.0
Sulfur (2)	-	147	-	-
Phos-phorus (macro) (2)	111	99	-	124
Vitamin C (2)	-	-	-	-
Vitamin B1 (thiamin) (2)	-	-	-	0.25
Vitamin B2 (ribo-flavin) (2)	-	-	-	-
Vitamin B3 (niacin) (2)	-	-	-	-
Alpha-tocoper-ol (Vit E) (2)	-	-	-	45

(1)g/100g = grams/100 grams dry material
(2)mg/100g = milligrams/100 grams dry material
Macro = ? (Came from translated chart)
Micro = ? (Came from translated chart)
*Valores calculados por diferenca.
Taken from: Rogez, 2000, Table 22, p. 158; Translations from Greater Continents do Brasil, Ltda.

Anthocyanins: The natural red pigment in *Euterpe oleracea* comes from the anthocyanins found naturally in the fruit. Red dyes in the food industry are very important, since certain synthetic dyes are restricted and/or banned. The amount of anthocyanin found in the acai fruit was about 336mg/100 grams (calculated as cyanidin 3-glucoside). This is higher than the amount found in plums (*Prunus salicin) and* in anil trepador fruits *(Cissus sicyoides*). *More* toxicological and stability studies are needed before this natural pigment is approved as a natural dye (laderoza et al, 1992). Anthocyanins are known to have antioxidant properties that are health promoting. Antioxidants are important due to their free radical scavenging activity. Free radical damage has been linked to the aging process and almost every chronic degenerative disease including heart disease, arthritis, and cancer (Pizzorno & Murray, 1999). ORAC units (oxygen radical absorption capacity) can be used as a measure to show antioxidant activity.

Fatty Acid Content: The oil from fruit mesocarps contains the following fatty acid percentages (taken from Lubrano et al, 1994):

C16:0 Palmitic acid	22% (saturated fatty acid)
C16:1 Palmitelaidic acid	2% (*trans* fatty acid)
C18:0 Stearic acid	2% (saturated FA)
C18:1 Oleic acid	60% (mono-unsaturated FA)
C18:2 Linoleic acid	12% (polyunsaturated FA)
C18:3 Linolenic acid	<0.5% (polyunsaturated FA)
C:20 Arachidic acid	2.5% (energy source)

Note: C:20 is listed as C:18 in Lubrano et al, 1994, and is most likely a mistake, since C18:0 was already listed.

The oil from the acai fruit is considered good fat because it is 60 % mono-unsaturated and 12% polyunsaturated. The total amount of saturated fat is 24%, along with the amount of *trans* fatty acids (which behave like saturated fats and raise cholesterol levels) at 2%. Both saturated fat and *trans* fatty acids are considered bad fats because at high levels they increase the risk of atherosclerotic vascular disease due to the fact that they increase cholesterol levels in the blood. Alpha-linolenic acid, an omega-3 polyunsaturated fatty acid, is very important for health, along with its counterpart gamma-linolenic acid. Both linolenic acids are involved in many disease processes

and are not consumed at high enough levels by most people in the United States (Pizzorno & Murray, 1999).

Quality Control: Recommendations for the quality standards for the drink have been officially adopted by the Brazilian government, but have not been obtained, due to difficulty in retrieving information from Brazil. Poor preservation of the fruit after picking and of the drink after preparation is a common problem. The microbial contamination (bacteria, fungi, and yeast) is a factor responsible for their fast deterioration. The microbial contamination originates from the trees, the fruits rubbing together after picking, from the soil (fecal coliforms), and depends on the time between the picking and marketing. Almost half of the anthocyanins degrade 2 days after fruit picking. The importance of peroxidase and polifenoloxidase activities constitute a second factor influencing the rancidity problems and the accelerated browning of the drink (Rogez, 2000). There are a number of ways to improve shelf-life and merit brief mentioning. Gaseous sulfiting [15 ppm(parts per million), 10 — 60 minutes] and blanching (80 degrees Celcius/10 seconds) of the fruits improves sanitary quality and helps preserve the anthocyanins. Pasteurization of the drink by an HTST process (87.5 degrees Celsius/1 min) permits the inactivation for the peroxidase and the polifenoloxidase, as well as the complete destruction of pathogens, fungi, and yeasts. The addition of citric acid facilitates the treatment and partially protects the anthocyanins (Rogez, 2000).

Note on contents of this summary: The original research was done in Brazil and most is unobtainable to date. A number of studies have been done at the universities, but problems with retrieving studies from Brazil remains a road block. Two very important studies that review the chemical composition and nutritive value of acai were not obtained in order to verify information that would be useful for making claims. The 2 most important studies are Rogez et al., Biochemical and Technical Studies on Acai, no year included and Calzavara, 1972. A summary of the Rogez study includes information on nutrient composition reviewed for 10 studies, but no units are listed by the amounts, therefore this data is unusable. The information that was obtained was mostly translated from Portuguese, where valuable information and more detailed descriptions could have been lost.

Only referenced material was included in this summary. HTST was not described, and therefore is not defined in the summary.

Update (3/5/01): GCB will be gathering studies and translating them for AIBMR, including the important studies mentioned in the note above.

References:

Pizzorno & Murray. Textbook of Natural Medicine. 2nd Edition. Volume 1. Churchill Livingstone. New York. (1999) p. 136-140, 900.

Cavalcante, Paulo B. Edible Palm Fruits of the Brazilian Amazon. Translated into English by Dennis Johnson, University of Houston, Texas. Arboretum Amazonicum 5th Decade Iconography of the Most Important Spontaneous and Cultivated Plants from the Amazon Region. (1988) p. 93-96.

Greater Continents do Brasil, Ltda. Technical and Marketing materials. (2000).

Iaderoza M, Baldini VLS, dos Santos Draetta I, and Bovi MLA. Anthocyanins from fruits of acai (*Euterpe oleracea*, Mart.) and Jucara (*Euterpe edulis*, Mart.). Trop. Sci. 1992;32:41-46.

Lubrano C, Robin JR, Khaiat A. Composition en acides gras, sterols et tocopherols d huiles de pulpe de fruits de six espeices de palmiers de Guyane. Oleagineux. Fevrier 1994;49(2):59-66. (Note: Article is in French with an English abstract)

Miret (Muniz-Miret) N, Vamos R, Hiraoka M, Montagnini F, Mendelson RO. The economic value of managing the acai palm (*Euterpe oleracea,* Mart.) in the floodplains of the Amazon estuary, Para, Brazil. Forest Ecology and Management. 1996;87:163-173.

Plotkin MJ & Balik MJ. Medicinal uses of South American palms. Journal of Ethnopharmacology. 1984;10:157-179.

Rogez, Herve. Acai: Prepario, Composicao e Melhoramento de Conservacao. Univerisidade Ferderal de Para, Belem, Brasil. EDUFPA (2000) p. IX-X. Table 22, page 158. www.acai.br. (Note: Portuguese book with an English abstract.)

Rogez H, Pascal S, de Souza JNS, Aquino AR, and Buxant R. Acai: Biochemical and technical studies on acai (*Euterpe oleracea*, Mart.). Dept de Engenharia Quimica - Centro Technol gico, Univeristy of Para, Belem, Brasil. Unidade de Bioquimica da Nutri o —Univerisida Cat lica de Louvain, Belgica. (Note: Summary excerpted from a study with missing references, journal name, volume, number, or year, along with a chart that reviews 10 researchers summary of nutrient composition without units and some letters not printing out in the text which may lead to misspellings and confusion above. Rogez, 2000, table 22, includes the units that were missing from the chart mentioned above.)

[redacted]

From: "Greater Continents" <gcbltda@terra.com.br> Save Address - Block Sender
Reply-To: "Greater Continents" <gcbltda@terra.com.br>
To: aibmr@hotmail.com Save Address
Subject: **Açaí Production Update: First batch missed mark**
Date: Mon, 11 Dec 2000 16:29:11 -0200

Açaí Production Update: First batch missed mark

On Tuesday, Nov .5, the first full batch of Açaí dehydrate became available. We suffered delays in delivery of almost two months, due to corporate restructuring of the supplier, which has caused much lost time and opportunity.

Although, after testing, the new batch proved to have better color and flavor than anticipated it exhibited a slight graininess that is not characteristic of Açaí and which was not present in the previous test lot. This characteristic makes the present batch inappropriate for sampling and demo uses but is a problem that can be resolved in future production.

Açaí production is a particularly unforgiving sequence of events due to enzymes and a proportionally high load of fermenting agents on the fruit skin compared to the quantity of pulp removed from the fruit. For this reason, Açaí production was traditionally limited to local and immediate consumption. In the early 1990's flash freezing was introduced to the production areas and the domestic production gained national distribution capabilities. In 2000 GCB developed its dehydrated Açaí which is expected to open the international market and which is also enjoying intense domestic interest.

Açaí frozen fruit pulp must be maintained at a temperature of -5° centigrade or less. At higher temperatures the enzymes and fermenting agents become active and change the characteristics of the fruit pulp. One effect is the creation of insoluble compounds, the grit mentioned above, which is evident with this last batch. These insolubles were encountered in the first batches of Açaí dehydrate (from two processors) and were found to be caused by the thawing of the pulp during the preparation for dehydrating. We resolved this problem by presenting the Açaí frozen material in a manner that did not require pre-thawing before dehydrating and succeeded in creating an Açaí that re-hydrated very successfully and met our quality requirements of color, texture and flavor.

The unfortunate situation with the present batch was caused by the thawing of the product (above -5° C) at the pre-transportation storage point. Electrical supply was lost and the container warmed. After the power returned the container was inspected. It was believed that thawing had not occurred and the product was shipped and sold to GCB and other customers. However, it is now apparent that thawing did occur which resulted in the current unacceptable batch. Our dehydrator also reported that the frozen Açaí did not meet physical specifications (not flat plates of frozen pulp but curved and malformed) which, we now realize, confirms the warming of the frozen pulp.

Prevent this from occurring in the future GCB and the MA ltda. are augmenting manufacturing procedures requiring monitoring of product temperature from producer to dehydrator.

Our goal of providing this exceptional fruit in a practical, consistent and quality form remains firm. And all steps necessity to accomplish this are being taken.

[redacted]

Although we do not have Açaí dehydrate available at this time a second batch is expected late December to early January.

This brings me to the second related topic: Uninterrupted production capacity .

A robust production capability is a necessity to meet market demands and justify the investment required to introduce a new product. Our supplier of Açaí is the only company who has a managed year around production capability of Açaí. Other producers rely upon two harvest of Açaí per year, one of excellent quality and volume, the second lower quality and volume. With this supplier we have the benefits of year around production and the best of the natural harvest seasons. The down side of the equation is that if they have delays we could be impacted. GCB has been assured that cause of the recent delay was a one time event which will not be repeated in the future. Additionally, a São Paulo warehouse has been established where reserve product will be stored.

Dehydrators: diversification, back up service suppliers.

The dehydrator we have been using is the best in Brasil, They work for the top international and domestic companies, whose products range from beverages, to soups and meals. An operational shutdown has commenced to upgrade and expand their production facilities during this slow period in Brasil (Dec-Feb). They will not be available to dehydrate until March.

Another dehydrator, closer to the Açaí production area and a specialist in fruit dehydration, is on its second test of our Açaí. This facility is newer and more flexible in regards to lot sizes. I will visit the facility during the holidays and expect to have the first production lot from them in early January.

I have also inspected a third dehydrator, in the SP area but was not sufficiently impressed with the facility or management to attempt Açaí with them. They may be fine for other products who's production specifications are not as critical.

This combination of year around production and multiple dehydrating facilities will assure that sufficient quality product will be available whenever needed.

Summary:

We do not have viable samples to distribute at this time.

I am preparing for a new batch of Açaí dehydrate, from an alternative producer, and expect delivery no later than mid January.

The reasons for this unfortunate delay are being addressed to assure consistency, quality and prompt deliveries in future.

- The first step is to set verification technology that confirms production, storage and transportation parameters are maintained.

- Second; set dehydrating parameters and methodology to assure production consistency from all dehydrators.

Diversification of service providers will help assure consistent availability .

Best regards,

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Chemical Composition of the Pulp of Açaí
commissioned by
Greater Continents do Brasil Ltda.

The fruit of Açaí has received increasing attention by the general population and by the scientific community because of the biological benefits that are attributed to it. However, little analytic data about its nutrient composition have been recently published in the scientific literature. Although from information divulged in various media; newspapers, magazines and diverse internet sites, Açaí is purported to be a food of high energy value and rich in micro-nutrients.

We have completed this study to present the available data regarding the chemical composition of Açaí and an critical evaluation of the reliability of the information presented by various sources.

There are various studies, quite old (1936-1977) (ref. not presented), with results that are significantly diverse, in particular those that refer to the levels of lipids, carbohydrates and dietary fiber in the pulp of the fruit. Calcium, iron and phosphorus, as well as the levels of vitamins were analyzed on only two occasions. All the data, despite the efforts of the authors to present effectively the composition of the fruit, are in question. The analyses were made in the past, with the use of methodology of less sensitivity and less specificity, and are today considered old fashioned and inadequate.

To aggravate the situation, there is no information regarding the origin of the fruits, if national or foreign, which could be a another factor that compromises the exactness of the results. The median results encountered were: protein 6.01% and the lipids varied between 17.92 - 24.75, total carbohydrates between 0.65 - 63.22% and the level of fiber between 30.51 and 74.59%, therefore the level of residual humidity would be about 40%.

From the decade of 1980, we encountered the work of AGUIAR and Colleges (1982) (1) that provided a chemical analysis of the macro-Nutrients, using methodologies considered adequate at that time. According to the authors, the level of proteins are 3.6%, lipids 2% and fibers 32.7% with the level of carbohydrates estimated by taking the difference between 100 and the sum of the total macro-nutrients included in water, of 57.4%. The water in the samples was 36.0%. To calculate the level of calories, expressed as kcal, multiply the levels of carbohydrates and proteins by 4 and the level of lipids by 9.

[redacted]

In relation to the data published up to 1977, we observed in the more recent works a lower level of protein and an enormous difference in relation to the levels of fat. While the works before the decade of 1970 reported values around 20% fat, AGUIAR and Colleges detected only 2%. A difference of the levels of carbohydrates is also notable because it becomes (is) important in the calculation of calories.

The data that was analyzed was published in the Table of Chemical Composition of Food, (Tabela de Composi o Qu mica dos Alimentos) edited by G. Franco, according to its various editions, of the 6th (1982) through the 9th (1992) (13,14). Because they carry older data, a major critique that is made is in respect to the analysis of the level of dietary fibers. This criticism is well taken, because the analytical methods to determine the level of dietary fiber were replaced in the decade of 1990 for others, more specific, that reduced the error made in the analysis of the level of crude fiber. (fibra bruta). Consequently, the concentration of carbohydrates available is diminished, with resulting changes in the calculation of the energy levels.

Therefore, it is likely, that the data published in the Table of G. Franco does not correspond to the real composition of the fruit and justifies an analysis of the fruit using methodologies recognized as more adequate.

Comments should be made in regard to the analysis of the micro-nutrients and vitamins. The technical advances of analysis s beginning in the decade of 1980, with the introduction of the methods of Atomic Absorption and Plasma Absorption, as well as high efficiency chromatographic methods, that permitted the analysis of minerals and isomers of major and minor vitamin activity. These advances make possible the obtaining of new analytical data and the creation of data banks to arrange Tables of the Composition of Foods, more accurately.

In 2000, a book by H. Rogez was published that studied intensely the fruit Acai including its chemical, technical and microbiological characteristics. Apparently, this book represents the one source of more recent data, yet published.

Analysis of 124 samples of A a resulted in the following data, expressed in a dry base.

Lipids :	52.64%	
Proteins:	10.05%	
Carbohydrates available:		2.96%
Elementary fiber:		24.22%
Ash:	3.09%	
K:	990 mg/100g	
Na:	76 mg/100g	
Ca:	309 mg/100g	
Mg:	178 mg/100g	
P:	147 mg/100g	
Fe:	20.59 mg/100g	
Zn:	17.30 mg/100g	
Vitamin B1:	0.25 mg/100g	
α-tocoferol;	45.00 mg/100g	

[redacted]

The analytical methodology used by this researcher may be considered adequate.

In a comparable analysis, we present here the analytical data of the pulp of freeze dried a a , with a residual humidity content of 3.39%. this analysis was done in the USA by Silliker Laboratories in January 2001.

Calories:	534 Kcal/100g
Lipids:	32.51%
Protein:	8.11%
Carbohydrates total:	52.2%
Dietary Fiber:	44.32%
Ash:	3.78%
Ca:	260 mg/100g
Fe:	4.4 mg/100g

We here comment on the data regarding composition divulged and available on the internet from diverse sites on the internet. Nutritional information about the fruit pulp of A a can be encountered on diverse sites that commercialize tropical fruit and in sites containing information for healthy sports diets, people who use health spas/gymnasiums.

We observed that the data retrieved was from basically two sources: 1) Tabela de Composi o de G. Franco, limited by the restrictions mentioned above. 2) Data published by Herv Rogez.

The data divulged on these sites should be viewed cautiously. The tables presented often contain gross errors, such as: the lack of the units of measure used to express a concentration, the exchanging of gram for milligram and the presentation of data without mention of source.

Regarding the level of anthocyanins in the Acai fruit, we encountered data in the order of 350-400 mg/100g of pulp (6-15), however its concentration can reach levels of 926 mg/100g of pulp during the period of the harvest.

All the values mentioned can be encountered in Tables 1-3, attached.

Tables 1-3 compare analytical data assembled from the literature consulted.

Table 1. Chemical Composition of acai (*Euterpe oleraceae Mart*) and calculation of energy (g/100 g of part consumable)

Humidity	Proteins	Lipids	Carbohydrates	Fibers	Calories	Minerals	Reference number
36.0	3.60	2.00	57.40	32.70	262	1.00	1
nd	3.80	nd	nd	nd	247	nd	2
nd	3.80	12.20	36.60	nd	247	nd	3
nd	13.00	17.00	nd	34.00	nd	nd	4
nd	5.00	12.00	12.00	16.00	240	nd	5
nd	13.00 mg	17.00	36.60/12.00	34.0mg	240	nd	6
nd	3.80	nd	nd	16.90	247	nd	7
nd	13.00 g	17.00 g	nd	34.00 g	nd	nd	8
41g	3.35	24.38 g	12.02 g	18.00	nd	1.25	10
Dry Mat.	10*	52.00 g	-	25 *	-	-	12
nd	3.8	12.20	35.60	nd	247	nd	13-14
nd	2.37	5.96	nd	nd	nd	nd	17
Dry Mat.	10.05**	52.64**	-	25.22**	-	3.00**	19

nd — not determined

Results expressed as g/100g of acai pulp in accordance with the source.

* Composition of juice, expressed as % of dry base

** Composition of pulp, expressed as % of dry base

Table 2. Composition of micronutrients and anthocyanins.

Calcium	Iron	Phosphorus	Magnesium	Sodium	Potassium	Anthocyanin	Source
118 mg	11.8 mg	58 mg	nd	nd	nd	nd	2
118 mg	11.8 mg	0.50 mg	nd	nd	nd	nd	3
286 mg	26.0 mg	227.0 mg	174.0mg	56.4 mg	932.0 mg	nd	4
9.43	nd	7.58	6.46	nd	34.0	400.mg	5
286 mg	26.0 mg	227.0 mg	174.0 mg	56.4 mg	932.0 mg	400 mg	6
118 ?	58 ?	nd	nd	nd	nd	nd	7
286 mg	26.0 mg	227.0 mg	174.0 mg	56.4 mg	932.0 mg	nd	8
nd	11.8 mg	nd	nd	nd	nd	nd	9
nd	11.8 mg	nd	nd	nd	nd	nd	11
nd	-	-	-	-	-	1 g	12*
118 mg	11.8 mg	0.5 mg	nd	nd	nd	nd	13-14
-	-	-	-	-	-	336 mg	15
50 mg	-	-	-	-	-	356.7-926.1 mg	16
309 mg	0.90 mg	33 mg	-	-	-	-	17
	20.6 mg	147 mg	178.0 mg	76.mg	990.0 mg	-	19**

nd — not determined

* Composition of juice, expressed as % of dry base

** Composition of pulp, expressed as % of dry base

Table 3. Composition of vitamins

B1	B2	C	Niacin	PP	E	Total Carotenes	Retinol	Source
nd	nd	nd	nd	nd	nd	900 mcg	150 mcg	1
360µg	10µg	9.0 mg	0.4 mg	nd	nd	nd	0µg	3
nd	nd	17.0 mg	nd	nd	45 mg	nd	nd	4
360µg	10µg	17µg	nd	400µg	45µg	nd	nd	6
11.8?	0.36 ?	0.01 ?	nd	nd	nd	nd	nd	7
nd	nd	17.0 mg	nd	nd	45 mg	nd	nd	8
-	-	-	-	-	45 mg *	-	-	12
360µg	10µg	9 mg	0.4 mg	ndnd	nd	nd	0µg	13-14
traces	nd	nd	nd	nd	nd	nd	traces	17
0.25 mg	-	-	-	-	45 mg	-	-	19

nd — not determined

* Composition of juice, expressed as % of dry base

** Composition of pulp, expressed as % of dry base

Literature consulted

1. AGUIIAR, J.P.L.; MARINHO, H.A.;REBELO, Y.S.; SHRIMPTON, R. — Aspectos nutritivos de algums frutos da Amaz nica, 10(4): 755-758, 1980
   a. Methods of analysis
   b. Humidity: oven dried 105 °C
   c. Protein: Kjeldahl, AOAC
   d. Lipids: ? Extraction solvent organic. (probable)
   e. Fiber: crude fiber
   f. Carbohydrates (by subtraction)
   g. Carotenoids: no information
   h. Eq. Retinal: considers 6 µg carotenes = 1µg Retinal

2.

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RESEARCH REPORT
RPN 5515B

April 26, 2002

CHALLENGE TEST OF A PRODUCT

Prepared for

Mr. Alex Schauss
AIBMR/Life Sciences
4117 S. Meridian
Puyallup, WA 98373

Alex@aibmr.com

Prepared by

Tracy Winter, B.S.
Senior Microbiologist

Ellen Vestergaard, M.S.
Program Manager

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Objective

The objective of this study was to conduct a preliminary challenge test to assess the microbiological stability of a product when challenged with one strain each of yeast, mold, lactic acid bacteria, *Salmonella*, and *Staphylococcus aureus*.

Applications

This study offers a screening of a product for potential spoilage organisms and two pathogens. It is appropriate to gather initial data about a product and/or to compare a number of product formulations during development.

Limitations

With only one strain of each challenge organism, there is a chance that the product will be resistant to growth by that strain but susceptible to other strains. If the challenge organisms grow in the control product, it will not be determined until the end of the study. This study is limited in time intervals, storage temperatures, and the scope of the report. The study does not predict the results beyond four weeks.

Materials and Methods

Test Product

A 3.5 kilogram resealabel foil bag of product labeled "Acai fruit-freeze dried" was received from the client on 2/23/01. Product was stored at ambient temperature until initiation of the study on 3/14/01.

Challenge Organisms

The product was challenged with freeze dried strains of *Aspergillus niger* (mold), *Zygosaccharomyces bailii* (yeast), *Lactobacillus fructivorans* (lactic acid bacteria), *Salmonella typhimurium*, and *Staphylococcus aureus* from the Silliker Research Culture Collection(SRCC). The number of viable cells or spores was verified by plate count methods.

Organism	SRCC Number
Aspergillus niger	1131
Zygosaccharomyces bailii	764
Lactobacillus fructivorans	464
Salmonella typhimurium	449
Staphylococcus aureus	713

Preparation of Test Samples and Storage

The product was aseptically divided into 6 sterile containers in 100-gram portions. One portion served as a negative control. The other portions were inoculated with one of the cultures at approximately 10,000 colony forming units per gram. After inoculation, the samples were mixed thoroughly and stored at 75°F.

Sample Analyses

The uninoculated control portion was analyzed for challenge organisms on days 0 and 28. Inoculated portions were analyzed on days 0, 7, 14, 21, and 28. A single 11-gram sample was taken from each portion at each interval and analyzed by plate count methods for challenge organisms.

Results and Discussion

The microbiological stability of a food product may be determined by challenging it with spoilage and pathogenic microorganisms. When the level of the challenge organisms does not increase during storage, the product formulation is resistant to microbial growth and is considered microbiologically stable.

Test results are shown in Tables 1 and 2. As the data show, the counts of yeast, mold, lactic acid bacteria, *Salmonella*, and *Staphylococcus aureus* did not increase in the control or inoculated portions of the product during storage. Thus, the Acai fruit-freeze dried product was microbiologically stable for at least 28 days when challenged with yeast, mold, lactic acid bacteria, *Salmonella*, and *Staphylococcus aureus* and stored at 75°F.

Table 1 Acai Fruit – Freeze Dried Non-inoculated Control Samples

Interval	Date	Yeast (cfu/g)	Mold (cfu/g)	Lactic Acid Bacteria (cfu/g)	*Salmonella* (cfu/g)	*Staphylococcus* (cfu/g)
Day 0	3-14	20	<10	20	<10	<10
Day 28	4-11	10	<10	20	<10	<10

cfu/g = colony forming units per gram

Table 2 Acai Fruit – Freeze Dried Inoculated Samples

Interval	Date	Yeast (cfu/g)	Mold (cfu/g)	Lactic Acid Bacteria (cfu/g)	*Salmonella* (cfu/g)	*Staphylococcus aureus* (cfu/g)
Day 0	3-14	470	44,000	3,400	190	44,000
Day 7	3-21	180	50,000	2,800	80	100
Day 14	3-28	10	10,000	580	170	50
Day 21	4-4	20	27,000	800	30	<10
Day 28	4-11	30	4,700	230	10	<10

cfu/g = colony forming units per gram

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RESEARCH REPORT

Mr. Alex Schauss
AIBMR/Life Sciences
4117 S. Meridian
Puyallup, WA 98373

Alex@aibmr.com

DATE: 4/26/02
REPORT NUMBER: 5515
SAMPLES RECEIVED: 2/23/01
SAMPLE DESCRIPTION: Acai fruit-freeze dried
SAMPLE STORAGE TEMPERATURE: 75°F

SHELF-LIFE STUDY TEST RESULTS

MONTH	DATE	AEROBIC PLATE COUNT (CFU/g)	YEAST (CFU/g)	MOLD (CFU/g)	LACTIC ACID BACTERIA (CFU/g)
0	3-14-01	---	20	<10	20
1	4-11-01	---	10	<10	20
2	5-9-01	1,500	30	40	10
3	6-6-01	50	<10	<10	10
4	7-4-01	750	<10	<10	<10
5	8-1-01	500	<10	<10	180
6	8-29-02	660	<10	<10	10
7	9-26-01	1,200	<10	<10	<10
8	10-24-01	360	<10	10	<10
9	11-21-01	<10	<10	10	<10
10	12-19-01	<10	<10	<10	<10
11	1-16-02	<10	<10	<10	<10
12	2-13-02	<10	<10	<10	<10

CFU/g = Colony Forming Units per gram

RESULTS AND DISCUSSION: The taste, odor and appearance of a food (organoleptic qualities) are the ultimate criteria used by consumers to judge a food's acceptability. These qualities begin to change as the microflora in the food- bacteria, yeast, and mold- grow and metabolize available nutrients. Organoleptic changes are generally not detectable until the microbial population is high. The number of organisms required to cause spoilage varies with the food item and the type(s) of microorganisms growing in it. Generally, however, the end of shelf-life is defined as 10,000,000 bacteria per gram, 100,000 yeast per gram or visible mold. As the data indicate, the counts of spoilage microorganisms did not exceed the pre-defined shelf-life limits during storage. Therefore, the shelf-life of the Acai Fruit-Freeze Dried product was at least 12 months stored at 75°F.

Tracy Winter
Tracy Winter, B.S.
Senior Microbiologist

Ellen Vestergaard
Ellen Vestergaard, M.S.
Program Manager

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1304 Halsted Street
Chicago Heights, IL 60411
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FOOD SAFETY • QUALITY • NUTRITION

CERTIFICATE OF ANALYSIS

COA No:	CHG-17077345-0
Supersedes:	None
COA Date	4/6/01
Page 1 of 2	

TO:
Ellen Vestergaard
Silliker Laboratory Research
160 Armory Drive
South Holland, IL 60473

Received From:	South Holland, IL
Received Date:	3/20/01

Location of Test: (except where noted)
Chicago Heights, IL

Analytical Results

Desc. 1: SAMPLE# AIBMR

Laboratory ID: 170547501
Condition Rec'd: NORMAL
Temp Rec'd (°C): 18

Analyte — Nutrition - Mandatory — **Test Date** 4/6/01 — **Loc.**

NUTRITIONAL ANALYSIS

Serving Size: 100 g

LABEL ANALYTES		ANALYTICAL DATA PER 100G	ANALYTICAL DATA PER SERVING	ROUNDED DATA PER SERVING	% DAILY VALUE
Calories		533.9	533.9	530	
Calories from Fat		292.6	292.6	290	
Total Fat	(G)	32.51	32.51	33	51
Saturated Fat	(G)	8.09	8.09	8	40
Cholesterol	(MG)	13.5	13.5	15	5
Sodium	(MG)	30.4	30.4	30	1
Total Carbohydrate	(G)	52.2	52.2	52	17
Dietary Fiber	(G)	44.23	44.23	44	176
Sugars	(G)	1.26	1.26	1	
Protein (F=6.25)	(G)	8.11	8.11	8	
Vitamin A	(IU)	1002	1002		20
Vitamin C	(MG)	<1.0	<1.0		*
Calcium	(MG)	260	260		25
Iron	(MG)	4.4	4.4		25
CONTRIBUTING ANALYTES					
Moisture	(G)	3.39	3.39		
Ash	(G)	3.78	3.78		
Beta Carotene	(IU)	<5	<5		
Retinol	(IU)	1002	1002		
Vit A % Beta Carotene		*			

* Contains less than 2% of the Daily Value of this nutrient

SUGAR PROFILE

Fructose	0.39	Glucose	0.76
Lactose	<0.10	Maltose	0.11
Sucrose	<0.10		

[redacted]

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CERTIFICATE OF ANALYSIS

COA No:	CHG-17077345-0
Supersedes:	None
COA Date	4/6/01
Page 2 of 2	

TO:
Ellen Vestergaard
Silliker Laboratory Research
160 Armory Drive
South Holland, IL 60473

Received From:	South Holland, IL
Received Date:	3/20/01

Location of Test: (except where noted)
Chicago Heights, IL

Analytical Results

SATURATED FATTY ACID	FORMULA	%
Butyric	4:0	<0.1
Caproic	6:0	<0.1
Caprylic	8:0	<0.1
Capric	10:0	<0.1
Undecanoic	11:0	<0.1
Lauric	12:0	0.1
Tridecanoic	13:0	<0.1
Myristic	14:0	0.2
Pentadecanoic	15:0	<0.1

SATURATED FATTY ACID	FORMULA	%
Palmitic	16:0	24.1
Margaric	17:0	0.1
Stearic	18:0	1.6
Nonadecanoic	19:0	<0.1
Eicosanoic	20:0	<0.1
Behenic	22:0	<0.1
Tricosanoic	23:0	<0.1
Lignoceric	24:0	<0.1

MONOUNSATURATED FATTY ACID	FORMULA	%
Tridecenoic	13:1	<0.1
Myristoleic	14:1	<0.1
Pentadecenoic	15:1	<0.1
Palmitoleic	16:1	4.3
Margaroleic	17:1	0.1
Oleic	18:1C	56.2
Elaidic	18:1T	<0.1
Gadoleic	20:1	<0.1
Erucic	22:1	<0.1
Nervonic	24:1	<0.1

POLYUNSATURATED FATTY ACID	FORMULA	%
Linoleic	18:2	12.5
Linolenic	18:3	0.8
Gamma Linolenic	18:3G	<0.1
Eicosadienoic	20:2	<0.1
Eicosatrienoic	20:3	<0.1
Homogamma Linolenic	20:3G	<0.1
Arachidonic	20:4	<0.1
Eicosapentaenoic	20:5	<0.1
Docosadienoic	22:2	<0.1
Docosahexaenoic	22:6	<0.1

Total Monounsaturated Fatty Acid	60.60
Total Saturated Fatty Acid	26.10
Total Polyunsaturated Fatty Acid	13.30

Douglas J. Winters, M.S. Laboratory Director

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CERTIFICATE OF ANALYSIS

COA No:	CHG-17076720-0
Supersedes:	None
COA Date	4/2/01
Page 1 of 1	

TO:
Ellen Vestergaard
Silliker Laboratory Research
160 Armory Drive
South Holland, IL 60473

Received From:	South Holland, IL
Received Date:	3/20/01

Location of Test: (except where noted)
Chicago Heights, IL

Analytical Results

Desc. 1: SAMPLE# AIBMR

Laboratory ID: 170547512
Condition Rec'd: NORMAL
Temp Rec'd (°C): 18

Analyte	Result	Units	Method Reference	Test Date	Loc.
Amino Acids Complete			USDA 6.011 (1986)	3/30/01	
Aspartic Acid	0.83	%			
Threonine	0.31	%			
Serine	0.32	%			
Glutamic Acid	0.80	%			
Glycine	0.39	%			
Alanine	0.46	%			
Valine	0.51	%			
Methionine	0.12	%			
Isoleucine	0.38	%			
Leucine	0.65	%			
Tyrosine	0.29	%			
Phenylalanine	0.43	%			
Lysine	0.66	%			
Histidine	0.17	%			
Arginine	0.42	%			
Proline	0.53	%			
Hydroxyproline	<0.01	%			
Cystine	0.18	%			
Tryptophan	0.13	%			

Douglas J. Winters, M.S. Laboratory Director

[redacted]

Adjusted for 25gram Serving Size 6045568 4/16/01 D.V.

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CERTIFICATE OF ANALYSIS

COA No:	CHG-17077345-0
Supersedes:	None
COA Date	4/6/01
Page 1 of 2	

TO:
Ellen Vestergaard
Silliker Laboratory Research
160 Armory Drive
South Holland, IL 60473

Received From:	South Holland, IL
Received Date:	3/20/01

Location of Test: (except where noted) Chicago Heights, IL

Analytical Results

Desc. 1: SAMPLE# AIBMR Euterpe deracea (freeze dried pulp)

Laboratory ID: 170547501
Condition Rec'd: NORMAL
Temp Rec'd (°C): 18

normal beverage serving size = 25gm

Analyte
Nutrition - Mandatory

Test Date Loc.
4/6/01

NUTRITIONAL ANALYSIS
Serving Size: 100 g

LABEL ANALYTES		ANALYTICAL DATA PER 100G	ANALYTICAL DATA PER SERVING	ROUNDED DATA PER SERVING (100g)	(25g)	% DAILY VALUE (100g)	% DV (for 25g Serving)
Calories		533.9	533.9	530	132.5		
Calories from Fat		292.6	292.6	290	72.5		
Total Fat	(G)	32.51	32.51	33	8.25	51	12.75%
Saturated Fat	(G)	8.09	8.09	8	2	40	10%
Cholesterol	(MG)	13.5	13.5	15	3.75	5	1.25%
Sodium	(MG)	30.4	30.4	30	7.5	1	.25%
Total Carbohydrate	(G)	52.2	52.2	52	13	17	4.25
Dietary Fiber	(G)	44.23	44.23	44	11	176	44
Sugars	(G)	1.26	1.26	1	0.25		
Protein (F=6.25)	(G)	8.11	8.11	8	2		
Vitamin A	(IU)	1002	1002			20	5%
Vitamin C	(MG)	<1.0	<1.0				*
Calcium	(MG)	260	260			25	6.25%
Iron	(MG)	4.4	4.4			25	6.25%
CONTRIBUTING ANALYTES							
Moisture	(G)	3.39	3.39	25gm / 0.8475			
Ash	(G)	3.78	3.78	0.945			
Beta Carotene	(IU)	<5	<5	<1.25			
Retinol	(IU)	1002	1002	250.5			
Vit A % Beta Carotene							

• Contains less than 2% of the Daily Value of this nutrient.

SUGAR PROFILE	100g	25g Serving		100g	25g Serving
Fructose	0.39	0.0975	Glucose	0.76	0.19
Lactose	<0.10	<0.025	Maltose	0.11	0.0275
Sucrose	<0.10	<0.025			

Sent to R.R. via fax 4/17/01

Note: divide all #s by 4 for a 25gm Serving Size (Beverage)

[redacted]

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CERTIFICATE OF ANALYSIS

COA No:	CHG-17077345-0
Supersedes:	None
COA Date	4/6/01
Page 2 of 2	

TO:
Ellen Vestergaard
Silliker Laboratory Research
160 Armory Drive
South Holland, IL 60473

Euterpe Oleracea
(Açaí)
freeze dried pulp

Received From:	South Holland, IL
Received Date:	3/20/01

Location of Test: (except where noted) Chicago Heights, IL

Analytical Results

SATURATED FATTY ACID	FORMULA	%
Butyric	4:0	<0.1
Caproic	6:0	<0.1
Caprylic	8:0	<0.1
Capric	10:0	<0.1
Undecanoic	11:0	<0.1
Lauric	12:0	0.1
Tridecanoic	13:0	<0.1
Myristic	14:0	0.2
Pentadecanoic	15:0	<0.1

SATURATED FATTY ACID	FORMULA	%
Palmitic	16:0	24.1
Margaric	17:0	0.1
Stearic	18:0	1.6
Nonadecanoic	19:0	<0.1
Eicosanoic	20:0	<0.1
Behenic	22:0	<0.1
Tricosanoic	23:0	<0.1
Lignoceric	24:0	<0.1

MONOUNSATURATED FATTY ACID	FORMULA	%
Tridecenoic	13:1	<0.1
Myristoleic	14:1	<0.1
Pentadecenoic	15:1	<0.1
Palmitoleic	16:1	4.3
Margaroleic	17:1	0.1
Oleic	18:1C	56.2
Elaidic	18:1T	<0.1
Gadoleic	20:1	<0.1
Erucic	22:1	<0.1
Nervonic	24:1	<0.1

POLYUNSATURATED FATTY ACID	FORMULA	%
Linoleic	18:2	12.5
Linolenic	18:3	0.8
Gamma Linolenic	18:3G	<0.1
Eicosadienoic	20:2	<0.1
Eicosatrienoic	20:3	<0.1
Homogamma Linolenic	20:3G	<0.1
Arachidonic	20:4	<0.1
Eicosapentaenoic	20:5	<0.1
Docosadienoic	22:2	<0.1
Docosahexaenoic	22:6	<0.1

Total Monounsaturated Fatty Acid	60.60	61% monounsaturated
Total Saturated Fatty Acid	26.10	26% saturated
Total Polyunsaturated Fatty Acid	13.30	13% polyunsaturated

Douglas J. Winters, M.S. — Laboratory Director

Received 4/5/01 D.V.

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CERTIFICATE OF ANALYSIS

COA No:	CHG-17076720-0
Supersedes:	None
COA Date	4/2/01
Page 1 of 1	

TO:
Ellen Vestergaard
Silliker Laboratory Research
160 Armory Drive
South Holland, IL 60473

Received From:	South Holland, IL
Received Date:	3/20/01

Location of Test: (except where noted)
Chicago Heights, IL

Servig — 100g

Analytical Results

Desc. 1: SAMPLE# AIBMR Eutrepe oleracea Acai (freeze dried pulp)

Laboratory ID: 170547512
Condition Rec'd: NORMAL
Temp Rec'd (°C): 18

Analyte	Result	Units	Method Reference	Test Date	Loc.
Amino Acids Complete			USDA 6.011 (1986)	3/30/01	
Aspartic Acid	0.83	%			
Threonine	0.31	%			
Serine	0.32	%			
Glutamic Acid	0.80	%			
Glycine	0.39	%			
Alanine	0.46	%			
Valine	0.51	%			
Methionine	0.12	%			
Isoleucine	0.38	%			
Leucine	0.65	%			
Tyrosine	0.29	%			
Phenylalanine	0.43	%			
Lysine	0.66	%			
Histidine	0.17	%			
Arginine	0.42	%			
Proline	0.53	%			
Hydroxyproline	<0.01	%			
Cystine	0.18	%			
Tryptophan	0.13	%			

4/11/01
amino acids are reported as
%, which is wt/wt (g/100g)
of sample per Tammy Baxa, Silliker

D.V.

Douglas J. Winters, M.S. Laboratory Director

SILLIKER LABORATORIES

Analytical Test Procedure

PROCEDURE NO.
ATP AMN08

EFFECTIVE DATE: April 10, 1996 REVISION NO: One REVISION DATE: December 18, 1996

AMINO ACID ANALYSIS BY ION-EXCHANGE CHROMATOGRAPHY WITH POST-COLUMN DERIVATION

A. *Principle:*
This method quantitatively determines amino acid content by hydrolysis with 6N hydrochloric acid followed by ion-exchange chromatography. O-phthaldehyde is used for post-column derivation and subsequent fluorometric detection.

B. *Scope:*
This procedure is applicable to ingredients, mixed feeds and any protein containing substance.

C. *Critical Points:*
1. Avoid excess evaporation time while drying samples. The loss of some amino acids may take place.

D. *Safety:*
1. Always follow the Chemical Hygiene Plan and established laboratory safety procedures for handling materials, cleaning up spills and disposing of wastes.
2. Read and observe all precautionary measures and hazards noted in the Material Safety Data Sheet for all chemicals used in this procedure.
3. Concentrated hydrochloric acid, potassium hydroxide and sodium hydroxide can cause severe chemical burns. Always wear protective equipment including gloves, safety glasses, lab coat, etc.
4. When preparing acid solutions, always add acid to water, not vise versa.
5. Perform dilutions in a fume hood to avoid breathing hazardous vapors.
6. Methanol is flammable. Caution must be taken at all times to keep the solvent away from sparks, heat and open flames. Know the location of the fire extinguishers in the area before proceeding with the test.
7. 2-Mercaptoethanol has a strong stench. Restrict all usage to the hood.

E. *Waste Disposal:*
1. The used buffer/OPA solution should be neutralized, as necessary, to 6.5-7.5 before disposing in the sanitary sewer system.

F. *Reagents & Chemicals:*
1. Water, HPLC grade, EM Science, EM WX0004-1 or in-house water purification system.
2. O-phthaldehyde, reagent grade, Anresco 0317.
3. Amino Acid standard solution, 2.5μmoles/mL, Sigma A9531.
4. Methanol, HPLC grade, Chempure 831-295 or equivalent.
5. Brij 35 solution, 30% (w/w), Sigma 430AG-6.

6. 2-Mercaptoethanol, (2-Hydroxyethylmercaptan), Sigma M-6250.
7. L-Norleucine, Sigma N-6877.
8. Pickering buffers, pH 2.2, 3.28, and 7.40, Pickering laboratories Na 220, Na 328, and Na 740.
9. Potassium hydroxide, pellets, Chempure 831-706.
10. Sodium hydroxide, pellets, Chempure 832-050.
11. Hydrochloric acid, 6 N volumetric solution, Chempure RH-155.
12. Ethylenediaminetetraacetic Acid EDTA Tetrasodium salt, hydrate, Sigma ED4SS.
13. Nitrogen source.
14. Boric acid, Chempure 830-314.
15. Norleucine Internal Standard - Weigh on an analytical balance to 0.1 mg, 0.1640 g of L-Norleucine. Transfer to 1000 mL volumetric flask. Add 250 mL HPLC water. Add 1 mL concentrated hydrochloric acid and mix. Make to volume with HPLC water, mix and sonicate. This solution will contain 1.25 µm/mL L-Norleucine. Refrigerate to avoid bacterial growth.
16. Amino Acid Standard Solution - Warm the vial of amino acid standard solution to room temperature. Pipet 5.0 mL into a 50 mL volumetric flask. Pipet 10.0 mL of 1.25 µm/mL L-Norleucine internal standard into the same 50 mL flask. Make to volume with HPLC water. Mix well and sonicate for several minutes. Transfer the standard into 4 mL Waters sample vials. Store at 0°C.
17. Potassium hydroxide solution, 50% - On a top loading balance, weigh 150 g of potassium hydroxide into a tared 1 liter Nalgene container. Dissolve with 150 g of deionized water. Stir as necessary. CAUTION! Exothermic reaction with caustic material. Allow the solution to cool to room temperature before usage.
18. Boric acid buffer - Weigh 122 g of boric acid into a tared 2000 mL beaker and add 1800 mL of HPLC water. Adjust the pH to 11.0 with 50% potassium hydroxide solution. Transfer the solution to a 4 liter glass jug and fill to volume (4 liters) with HPLC water and mix well. The final solution pH should be 10.4.
19. Pickering Buffer Mobile Phase -
    a. pH 3.28 - This buffer may be used as is from the bottle. Filter through a 0.45 µm filter membrane and degas prior to HPLC usage by vacuum under sonication.
    b. pH 7.40 - This buffer may be used as is from the bottle. Filter through a 0.45 µm filter membrane and degas prior to HPLC usage by vacuum under sonication.
20. Sodium hydroxide, 0.2 N - Weigh 16 g sodium hydroxide pellets into a 2 liter volumetric flask. Add approximately 1000 mL HPLC water and mix until the sodium hydroxide is dissolved. Weigh 0.5 g EDTA, add to the volumetric. Make to volume with HPLC water, mix and filter through a 0.45 µm filter membrane. Use plastic gallon jug as a reservoir for HPLC. Filter periodically.

21. O-phthaldehyde - Weigh 1.4 g of o-phthaldehyde (OPA) crystals into a 50 mL beaker. Add 20 mL HPLC grade methanol and sonicate until the crystals are dissolved. Add solution to a 2 liter volumetric flask containing approximately 1500 mL of boric acid buffer and mix. In a hood, add 4.0 mL 2-mercaptoethanol (stench!). Fill to volume with boric acid buffer and mix. Filter the solution through a 0.45 µm filter. Pour the filtered solution into two 1 liter Nalgene bottles and add 3.0 mL Brij-35 to each bottle. Cap the bottles with nitrogen and mix well. Refrigerate until needed. OPA solution is stable for approximately 1 week under these conditions. (may extend upto 2 weeks) 2 good. not with Cal

G. *Equipment & Apparatus:*

1. Waters model 712 B autoinjector or equivalent.
2. Waters model 6000 pump (2), Waters 2100 or equivalent.
3. Digital Pro 380 with Waters Expert software or equivalent.
4. Kratos FS-950 fluorometric detector or equivalent.
5. Kratos URS 051 post column pump or equivalent.
6. Fiatron column heater, Eppendorf CH-30 or equivalent.
7. Fisher Isotemp Oven, model 215 F or equivalent.
8. Savant Speed Vac Concentrator, model SVC-200H.
9. Savant refrigerated condensation trap, model RT-490.
10. Savant chemical trap, model SCT-120.
11. Savant disposable cartridge for acid vapor neutralization, model DC120A.
12. Precision direct drive vacuum pump, model Dd-310 or equivalent.
13. Vacuum gauge, Waters Pico-Tag work station or equivalent.
14. Glas-Col small pulsing vortexer, model S8216, Glas-Col PV6.
15. Beckman pHI40 Meter, Beckman 123118 or equivalent.
16. Mettler Analytical balance, model AE160 or equivalent.
17. Millipore solvent filtration apparatus, Waters 85116.
18. Interaction-Sodium loaded ion exchange column, with guard column, Interaction Chromatography AA511.
19. Bransonic Ultrasonic bath model 220.
20. Mettler top loading balance, model P-1000.
21. Pipetman, Gilson, 1 mL and 5 mL, Rainin P-1000 and P-5000.
22. Universal fit pipet tips, 1 mL to 5 mL, Rainin.
23. Plastipak syringe with Luer-Lok, 3 cc x 1/10 cc, BD 9585 or equivalent.
24. Syringe filters, polypropylene, Teflon, 0.45 micron, Nalgene 199-2045.
25. Magna Nylon 66 membrane 47mm diameter, 0.45 micron pore size, Fisher NO4SPC
26. Repipet II Dispenser, 5 mL, Fisher 13-687-62A.
27. Universal fit pipet tips, 200 - 1000 µl. VWR 53508-819.
28. Disposable culture tubes, 12 x 75 mm, borosilicate glass, VWR 60825-550 or equiv
29. Sample vial assembly, 4 mL, includes caps and PFTE septa, Waters 73018.

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30. Low volume insert with springs, plastic, for 4 mL sample vial, Waters 72163.
31. Firestone valve, rapid purge, Ace Glass Inc, 8766.
32. Culture tubes, disposable, 20 x 150 mm, screw cap, borosilicate glass, VWR 60826-28
33. Screw caps for disposable culture tubes, 20 mm OD, PTFE-faced liner, VWR 60828-57
34. Brinkman centrifugal mill, model ZM-1 (with 0.5 mm screen) or equivalent.

H. *Sample Preparation:*

1. It is essential that the sample be ground as fine as possible while keeping moisture loss t minimum. The sample should be ground through a Brinkman Centrifugal Grinding mill model ZM-1, or equivalent, using a 0.5 mm screen to obtain a fine grind.

I. *Procedure:*

1. Calibrate and zero the analytical balance.
2. It is helpful to have knowledge of the protein content of the sample before weighing for amino acid analysis. With this in mind, weigh the sample equivalent to 20 mg protein (0.1 mg) on an analytical balance. (Refer to the supplement for Free Amino Acid Determination) For a nearly pure sample, weigh approximately 38 mg. Record the we in a laboratory notebook. Quantitatively transfer the sample to a marked 20 x 150 mm screw top culture tube.
3. Using the Repipet II Dispenser, add 15 mL 6 N hydrochloric acid to each culture tube. Because of the grind and limited amount of sample taken, be certain to avoid any drafts that might cause a loss of sample from the culture tube.
4. In the hood, add 75 microliter of 2-mercaptoethanol to each culture tube (Note 1). Thi allows for better determination of the L-methionine peak.
5. Firestone (Note 2) each culture tube, alternating between nitrogen (10-12 psi) and vacu at least 5 times each. Do not allow the water in the sonicator to overheat.
6. Screw on the PTFE-faced cap while the sample is under nitrogen. To avoid injury fro broken tube, one may wish to wrap a cloth towel around the culture tube when tighteni the cap, as excessive force can break the tube.
7. Place the culture tubes in 110°C ± 2°C oven for 24 hours.
8. Assemble the Savant evaporation system. Start the power to the system at least 2 hour prior to usage as it takes this amount of time for the refrigeration unit to attain the fina working temperature of -92°C. Oil in the vacuum pump should have been thoroughly degassed. Do this as needed, by opening the gas ballast valve on the right side of the pump and switching the pump on. One hour is generally sufficient. Turn the pump o and close the gas ballast valve upon completion.
9. After 24 hours, remove the culture tubes from the oven and allow them to cool to roo temperature.
10. Add 5 mL of HPLC grade water to each culture tube. Screw on the cap and mix well.

11. Add 5 mL of Norleucine internal standard (1.25μm/mL) to each culture tube (Note 3).(At this point the analysis may be stopped until the following day if necessary. Store the culture tube at 0°C if you need to store overnight.)
12. Using a 1 mL pipetman, transfer 2 mL of hydrolysate into a marked 12 x 75 mm disposable culture tube.
13. Open the lid to the speed vac concentrator and place tubes containing the 2 mL of hydrolysate into positions around the rotor so that the load is well balanced.
14. Close the lid, open the vent and start the centrifuge. When the rotor has reached its operating rpms, close the vent on the vacuum gauge and start the vacuum pump. The evaporation process may take place overnight, if necessary. This would be so, if many samples were evaporated, starting late in the day (Note 4).
15. When samples are dry (vacuum gauge reads less than 500 millitorr), slowly open the vent to bleed air into the chamber. Turn the pump off and wait until the chamber has been completely vented before turning off the centrifuge, then remove the tubes.
16. Add 3 mL of Pickering sodium diluent 2.20 to each tube and momentarily sonicate prior t vortexing.
17. Attach a 0.45 μm filter to a 3 mL syringe. Filter the prepared hydrolysate into a marked mL vial containing a Waters low volume insert with spring, then place on 712B WISP autosampler tray.
18. HPLC Conditions:
    a. Degas all buffer and OPA solutions by sonication under vacuum. Place the buffer lines into appropriate buffer solution and OPA line into OPA solution. Equilibrate the column with buffers 3.28 with the flow rate at 0.5 mL/minute for at least 20 minutes.
    b. Set the sensitivity control on fluorometer to 450, range to 0.5, time constant to 1 second, background suppression to "lo". Make certain the shutter on the left side of the unit is pulled open and the lamp is on.
    c. Set the column heater temperature at 60°C. Monitor during the run.
    d. Start the OPA pump - set the flow rate to 0.50 mL/minute (Adjust downward as necessary).
    e. Place a standard in position #1 and #2 on WISP. Build multimethod and/or meth table and inject 20 μl of standard. Allow 60 minutes for run time. Observe resulting chromatogram. Inject a third time if chromatography is not satisfactory.
    f. Run a standard after every five samples. Updated response factors will be generated and used for subsequent injections.
    g. If the peak is outside the window, reprocess the samples and adjust the retention time in calibration table to match that of the sample. New corrected chromatogra will be printed and stored.
    h. Gradient elution using 2 buffers: Using Waters software, a satisfactory gradient h been established using 2 buffers for elution of amino acids. Pump Table follows:

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Analytical Test Procedure

PROCEDURE NO. ATP AMN08

EFFECTIVE DATE: April 10, 1996 REVISION NO: One REVISION DATE: December 18, 1996

PUMP TABLE: Standard Profile

Time	Flow	%A	%B	Curve No.	Total Flow
Initial	0.500	100	0	—	0.500
35.0	0.500	0	100	6	0.500
45.0	0.500	0	100	11	0.500
45.5	0.500	100	0	11	0.500

Where A = Pickering Buffer Na 328
B = Pickering Buffer Na 740

J. *Calculations:*

$$\textit{Response factor} = \frac{\textit{Amount amino acid (mg)} \times \textit{Area norleucine (internal standard)}}{\textit{Area amino acid}}$$

$$\textit{then} \quad \frac{RF \times \textit{Area amino acid sample}}{\textit{Area norleucine internal standard}} = \textit{Concentration of amino acid (mg)}$$

Since the sample volume determines the final concentration, concentration of amino acid times the sample volume = final concentration of amino acids.

$$\frac{\textit{Final concentration of amino acid (mg)}}{\textit{sample weight mg}} \times 100 = \textit{Amino acid \%}$$

K. *Notes:*

1. Mercaptoacetic acid may be used instead, if necessary. - if used Sub Brij-35 ~ to preserve Lys pea
2. The Firestone process consists of alternately evacuating and purging with nitrogen the acid-sample solution in a sonic bath. This will degas the solution and create an inert atmosphere above the acid thus minimizing oxidation of the amino acids during hydrolysi
3. Using a 5 mL pipetman, calibrate with room temperature water to 5.000 g ± 0.005 g.
4. With good vacuum, samples may freeze in the tubes. If so, after approximately 45 to 60 minutes, remove the tubes and warm hydrolysate in a beaker which contains hot water. Place tubes back into the rotor and continue evaporation.

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Analytical Test Procedure

PROCEDURE NO
ATP AMINO

EFFECTIVE DATE: April 10, 1996 REVISION NO: One REVISION DATE: December 18, 1996

L. *Validation:*

1. Validation is based on collaborative study with Degussa.

M. *Quality Control:*

1. Follow the standard quality assurance practices detailed in the Quality Assurance Manual.
2. A control standard (secondary standard) should be included in each run of samples. Case is currently used as a control.
3. Results of the control standard are to be recorded in the laboratory notebook.
4. Duplicate runs of the standard should not vary by more than 8%.
5. Notebooks are to be initialed and dated by the analyst performing the test.
6. Notebook entries are to be reviewed, understood, initialed and dated by another analyst in the department.

N. *References:*

1. JAOAC: Vol 65, No. 2, 1982, pp 496-497. Calculated Protein Efficiency Ration.
2. Degussa - Literature Digest for the Feedstuffs Industry - Amino Acid Analysis. GB Chemie/Anwendungstechnik Hanau Stadtteil Wolfgang, Fed. Rep. of Germany.
3. The Peptides, Vol. 4, Amino Acid Analysis of Peptides, Ch 5, pp 217-259, JR Benson, P.C. Louie and R.A. Bradshaw. Copyright 1981, Academic Press, Inc.
4. USDA Chemistry Laboratory Guidebook, G-41
5. JAOAC: Vol. 68, No. 5, 1985, pp 811-821. Sample Preparation for Chromatography of Amino Acids: Acid Hydrolysis of Proteins.

Submitted by:	Gregg Perri	Date:	May 1995
Reviewed by:	Perri	Date:	12/96
Approved by:	[signature]	Date:	December 18, 1996
Authorized by:		Date:	
Revised by:		Date:	
Distribution List:	Minnetonka - Lib, File		

Recommendations: See attachment for Free Amino Acid Determination.

File Name: ATP AMINO8

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FLORA RESEARCH

ANALYTICAL REPORT

DATE: October 5, 2001

CLIENT:

CLIENT'S SAMPLE ID: #001 Acai Powder

FLORA'S SAMPLE ID: 210823003

ANALYSIS: Quantitative Analysis of Sterols in Acai Powder

METHOD: High Resolution Gas Chromatography (HRGC)

RESULTS:

ANALYTE	PERCENT BY WEIGHT	
B-Sitosterol	0.044	= 0.44 mg/g
Campesterol	<0.003	= 0.03 mg/g
Sigmasterol	0.004	= 0.04 mg/g
Total Sterols	0.048	

REPORT PREPARED BY:

James P. Kababick, Director

10-05-01
Date

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http://www.floraresearch.com

[redacted]

Technical Bulletin No. C - 419

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Wareham, MA 02571
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"Bringing Good Science To Life" ™

$ORAC_{PE}$ $ORAC_{FL}$

ORAC ASSAY

The ORAC Assay was developed by *Cao et al*, and first reported in 1993: *"Cao G, Alessio HM, Cutler RG, Oxygen radical absorbance capacity assay for antioxidants. Free Radic Bio Medicine 1993;14:303-11"*. Modifications were made to automate the analytical procedure and were reported in the literature in 1995: *"Automated Assay of Oxygen Radical Absorbance Capacity with the COBAS FARA II, Guohua Cao, Carl P. Verdon, Alan H.B. Wu, Hong Wang, and Ronald L. Prior, CLINICAL CHEMISTRY, Vol. 41, No. 12, 1995"*.

From that point forward, the Automated ORAC Assay received extensive coverage and utilization, and as such, ORAC values have become commonplace in research and in the marketing of natural products. Brunswick Laboratories purchased two COBAS FARA II analyzers in 1997, replicated the automated method as developed by Cao, Prior, et al, and to date, has established an antioxidant database consisting of over 5000 points of ORAC data for fruits, vegetables, beverages, grains, functional/engineered foods, extracts, and other natural product sources.

During the course of analyzing a wide variety of samples over the past two years, the scientists at Brunswick Laboratories observed that there was variation in some of the ORAC results. After systematic investigation, we concluded that the variation was caused by the inconsistent quality of the β-Phycoerythrin(β-PE).

β-PE is a protein isolated from *Porphyridium cruentum*, without further purification, and as such, there is variability from lot-to-lot, which results in inconsistent reactivity to free radicals. In addition, protein binding between β-PE and some polyphenolic compounds present in the tested samples, is a problem which affects the initial fluorescence reading and lag phase, and consequently affects the ORAC results.

Dr. Boxin Ou and Ms. Maureen Hampsch-Woodill of Brunswick Laboratories, working with Dr. Ronald Prior of the USDA, introduced a new fluorescence probe, **fluorescein**, which has been tested with several hundred samples, in side-by-side comparison with β-Phycoerythrin. We found that unlike β-PE, **fluorescein** does not interact with the tested samples, and being a synthetic compound, fluorescein has no measurable variability from lot-to-lot. Most importantly, samples tested multiple times under the same conditions maintain consistent and repeatable results.

The noticeable difference between the two fluorescence probes is that the ORAC values are consistently 1.5 to 3 times greater with **fluorescein**, compared to β-PE. We concluded the discrepancy is due to **fluorescein** being less susceptible to free radical attack than β-PE, so there is more *free radical induced oxidation* of the antioxidant sample before oxidation of the **fluorescein** occurs.

The development of the ORAC Assay using **fluorescein** as the fluorescence probe has been conducted in cooperation with the developers of the original automated ORAC Assay, where β-PE was utilized as the fluorescence probe. Based on the extensively mechanistic studies, both parties look to the **fluoroscein** based ORAC assay as being the new standard ORAC procedure. We are in the transition period of adopting **fluorescein** as the standard probe and will distinguish the two ORAC assays by using the subscripts PE for phycoerythrin, and FL for **fluorescein** --$ORAC_{PE}$ and $ORAC_{FL}$.

Brunswick Laboratories does not want the transition to be a difficult move for our customers. We ask that if you are doing comparisons, or decision making, whereby a comparison of results between "old and new" causes difficulties, ***please contact us*** so that we can achieve an "apples to apples" comparison for you. Brunswick will look at each request on its' individual merit, and **minimize the trauma and costs to the best of our ability.** It's unfortunate that progress is sometimes painful—when it was discovered that the world is round instead of flat, even though it was progress, it caused a great deal of disruption for the mapmakers. We want to minimize the disruption to our valued customers for this improvement in the ORAC Assay.

Note to those using ORAC values in the marketing and/or comparison of products: Brunswick Laboratories recommends that "absolute" numbers/values not be used, because with the "standard deviation", the number/value will not always be exactly the same. Terms such as "– in the range of 'x'–" are more appropriate.

BRUNSWICK LABORATORIES
6 Thacher Lane • Wareham, MA 02571 • USA
Telephone 508-291-1830 Facsimile 508-295-6615

FOR IMMEDIATE RELEASE

August 02, 2001
Wareham, MA USA

" Bringing Good Science to Life"

Brunswick Laboratories: Scientists at Brunswick Laboratories announced today that they have developed a novel assay to measure the antioxidant activity of the oil soluble antioxidants--such as vitamin E-- found in foods and physiological fluids. This development greatly enhances the ability to provide a complete profile of all foods--fruits, vegetables, grains, nuts, juices/beverages--for their total antioxidant capacity. To date, the focus has been primarily on the water soluble antioxidants--such as vitamin C--and measured with an assay developed by the scientists at the USDA. Brunswick Laboratories is thought to have the largest data base in the world for this type data--over 5,000 ORAC (Oxygen Radical Absorbance Capacity) analyses to date. ***Antioxidants*** and ***anti-aging*** are often linked together, as antioxidants are thought to slow the aging process.

Jim Nichols, president of Brunswick Laboratories, points out that this assay is a milestone toward the total antioxidant profile. With this new assay, coupled with the original ORAC assay, the quantity per serving, or quantity by unit of weight or volume, of water soluble and oil soluble antioxidants that are available for use in the body, can be determined. These same two assays can be used to determine the antioxidant capacity of physiological fluids after a person and/or a research animal has been fed a defined profile of sources(foods) of antioxidants.

There is much being studied and written in the scientific world about antioxidants, phytochemicals, and nutraceuticals, with a tremendous amount of research underway to determine the bioavailability of the specific antioxidants from specific sources, and the role that these specific antioxidants may play with specific diseases. Brunswick's new assay will be a very important tool in this research process. It can also be a tool in the quality control of ingredients used in health supplements by providing fingerprints of both the water soluble and oil soluble antioxidant sources. Brunswick has already been able to define the differences in the antioxidant capacity of natural vitamin E versus synthetic vitamin E.

For copies of the assay manuscript, or for questions pertaining to the assay, please e-mail Brunswick at: mail@brunswicklabs.com.

CONTACT: James G. Nichols Brunswick Laboratories Wareham, MA USA
Telephone: 508-291-1830 Facsimile: 508-295-6615 e-mail: mail@brunswicklabs.com

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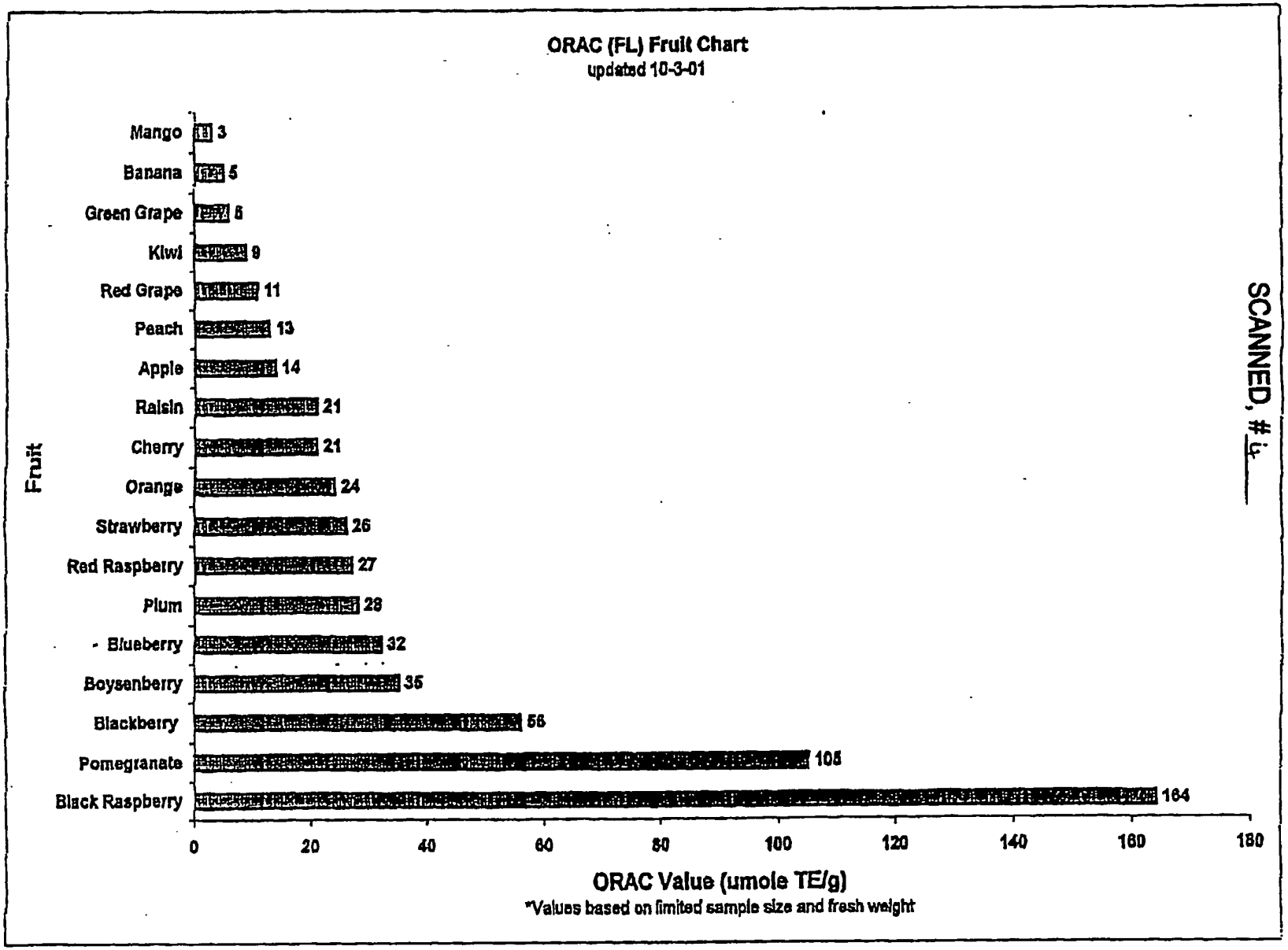
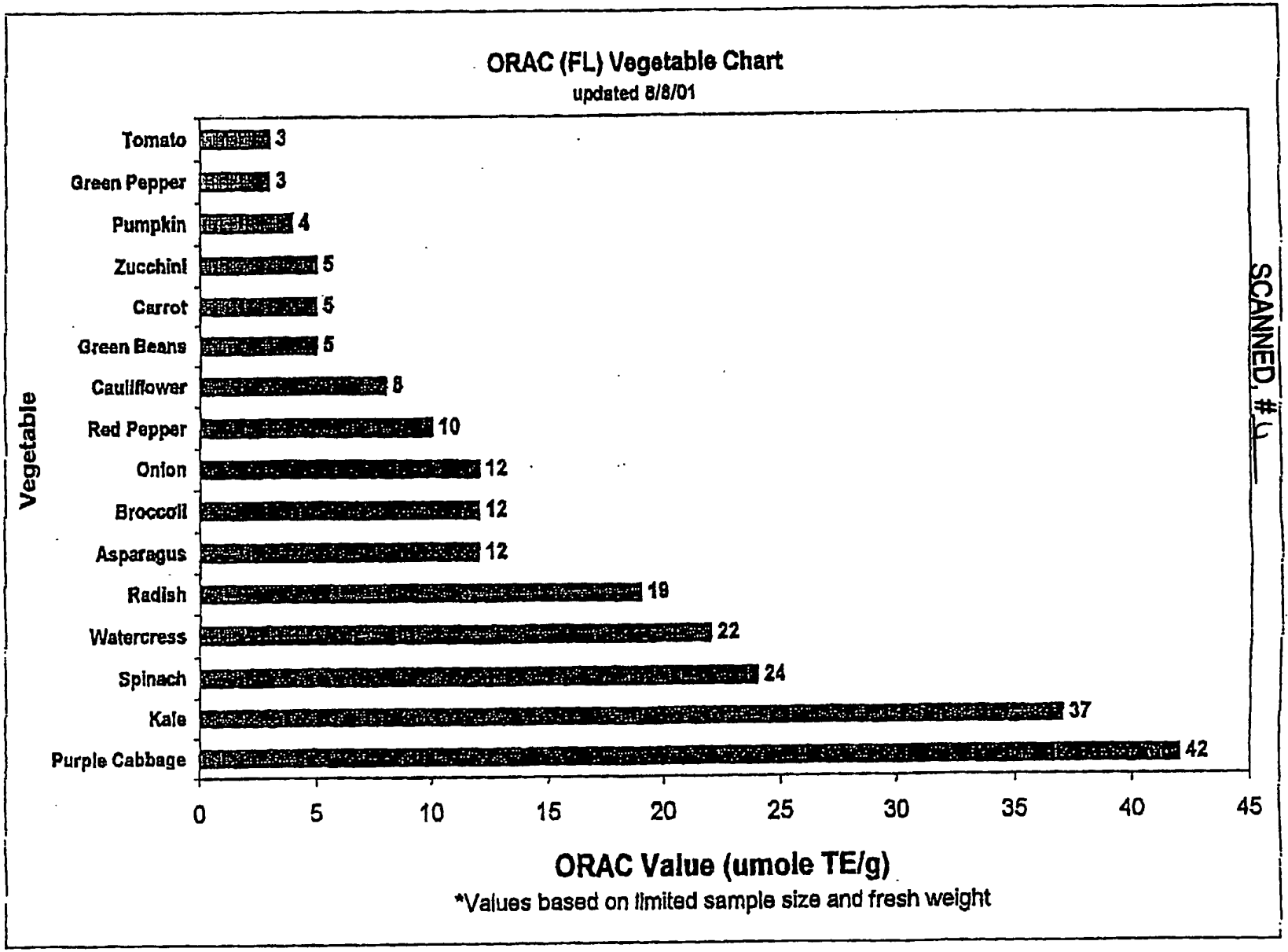
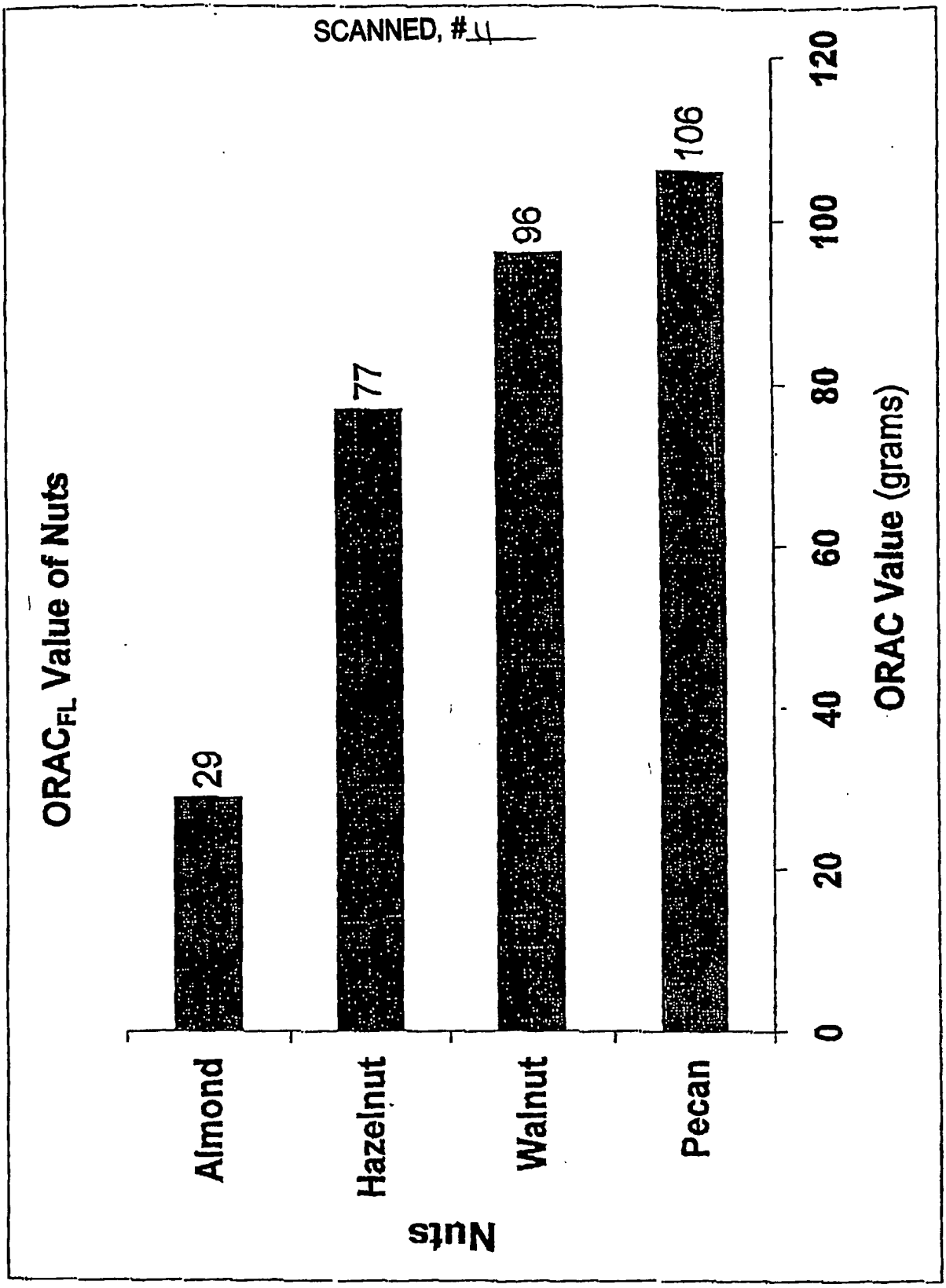
Compounds	ORAC (micromoleTE/g)
α–tocopherol (vitamin E)	1160
α–tocopherol acetate (or vitamin E acetate)	0
Vitamin C (L-ascorbic acid)	5110

[redacted]




ORAC (FL) Vegetable Chart
updated 8/8/01
Vegetable
Tomato 3
Green Pepper 3
Pumpkin 4
Zucchini 5
Carrot 5
Green Beans 5
Cauliflower 8
Red Pepper 10
Onion 12
Broccoli 12
Asparagus 12
Radish 19
Watercress 22
Spinach 24
Kale 37
Purple Cabbage 42
0 5 10 15 20 25 30 35 40 45
ORAC Value (umole TE/g)
*Values based on limited sample size and fresh weight


ORAC$_{FL}$ Value of Nuts
29
77
96
106
Almond
Hazelnut
Walnut
Pecan
Nuts
0
20
40
60
80
100
120
ORAC Value (grams)

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Report for American Institute for Biosocial and Medical Research, Inc.

Sample ID	Brunswick Lab ID	$ORAC_{FL}$* (µmoleTE/g)
Acai Sample 2/5/2002	02-0104	442

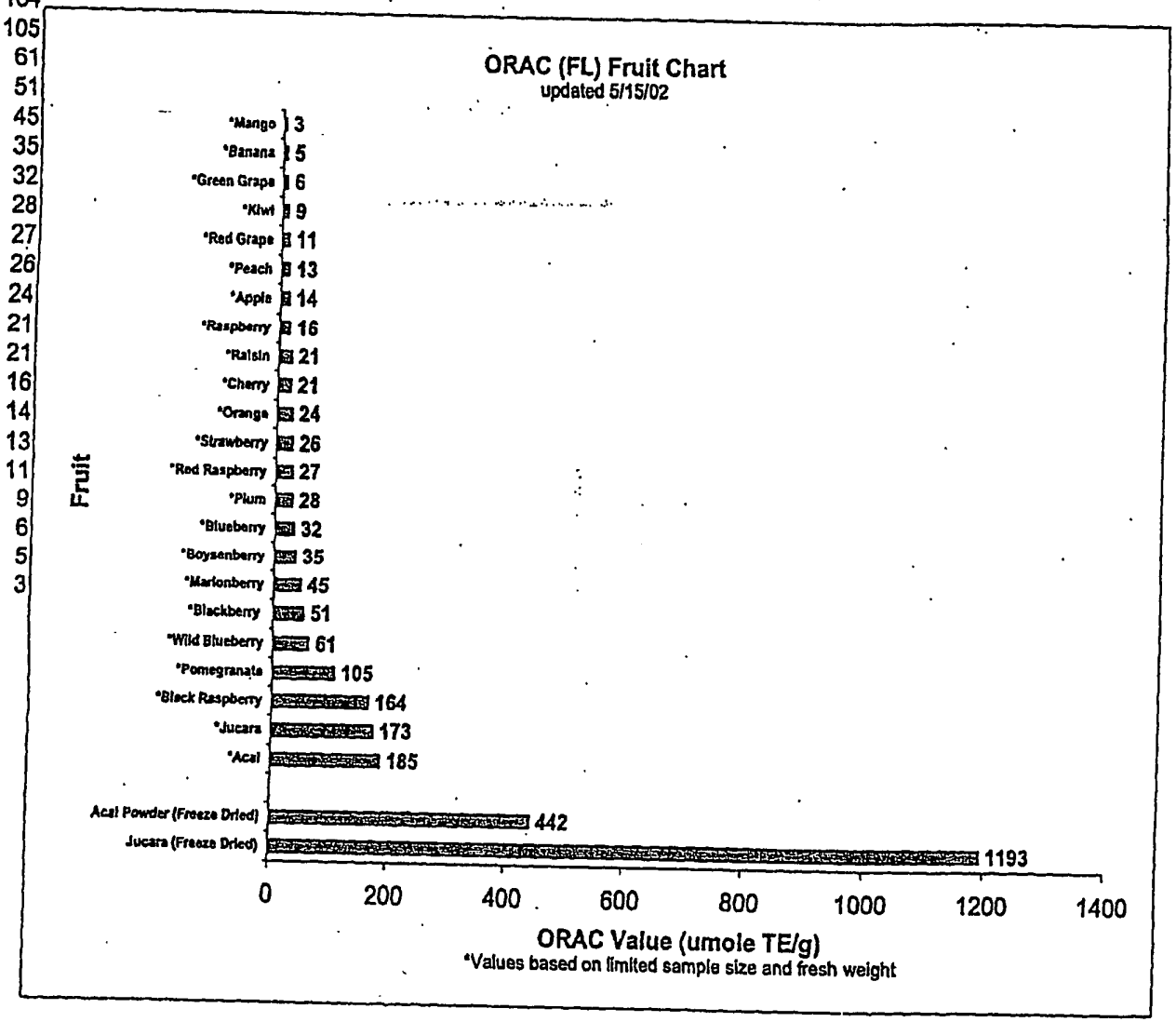
*The $ORAC_{FL}$ analysis, which utilizes Fluorescein as the fluorescent probe, provides a measure of the scavenging capacity of antioxidants against the peroxyl radical, which is one of the most common reactive oxygen species (ROS) found in the body.

Trolox, a water-soluble Vitamin E analog, is used as the calibration standard and the ORAC result is expressed as micromole Trolox equivalent (TE) per gram.

Testing performed by J. Flanagan.

B-821 / 2-7-02

Jucara (Freeze	1193
Acai Powder (I	442
*Acai	185
*Jucara	173
*Black Rasp	164
*Pomegran	105
*Wild Blueb	61
*Blackberry	51
*Marionberr	45
*Boysenber	35
*Blueberry	32
*Plum	28
*Red Raspl	27
*Strawberry	26
*Orange	24
*Cherry	21
*Raisin	21
*Raspberry	16
*Apple	14
*Peach	13
*Red Grape	11
*Kiwi	9
*Green Gra	6
*Banana	5
*Mango	3



UNIVERSIDADE DE SÃO PAULO
Faculdade de Ciências Farmacêuticas
Departamento de Alimentos e Nutrição Experimental
Laboratório de Análise de Alimentos

Relatório de análise

Amostra: aproximadamente 1 kg polpa de açaí congelada, recebida em 23 de abril de 2002.

Umidade: determinada em estufa à vácuo a 70°C, conforme metodologia descrita pelo Instituto Adolfo Lutz (1976).

Antocianinas totais: o teor foi determinado pelo método espectrofotométrico descrito por Francis & Fuleki, (J. Food Sci., v.33, p. 72-77, 1968) na amostra liofilizada e o resultado foi recalculado para a polpa integral)

Resultado:

Umidade na polpa de açaí (%)	85,37 ± 0,14
Umidade residual na polpa liofilizada (%)	1,06
Antocianinas totais (mg/ 100 g polpa) :	239,32 ± 0,74

Raw pulp
Residual humidity

São Paulo, 15 de maio de 2002

Profa. Ursula M. Lanfer Márquez

Açaí = Juçara

Av. Prof. Lineu Prestes, nº 580, Bloco 14 - Cidade Universitária - CEP 05508-900 - São Paulo - SP
Fone: (011) 3818-3684/3712 - Fax (011) 3815.4410

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Departamento de Alimentos e Nutrição Experimental
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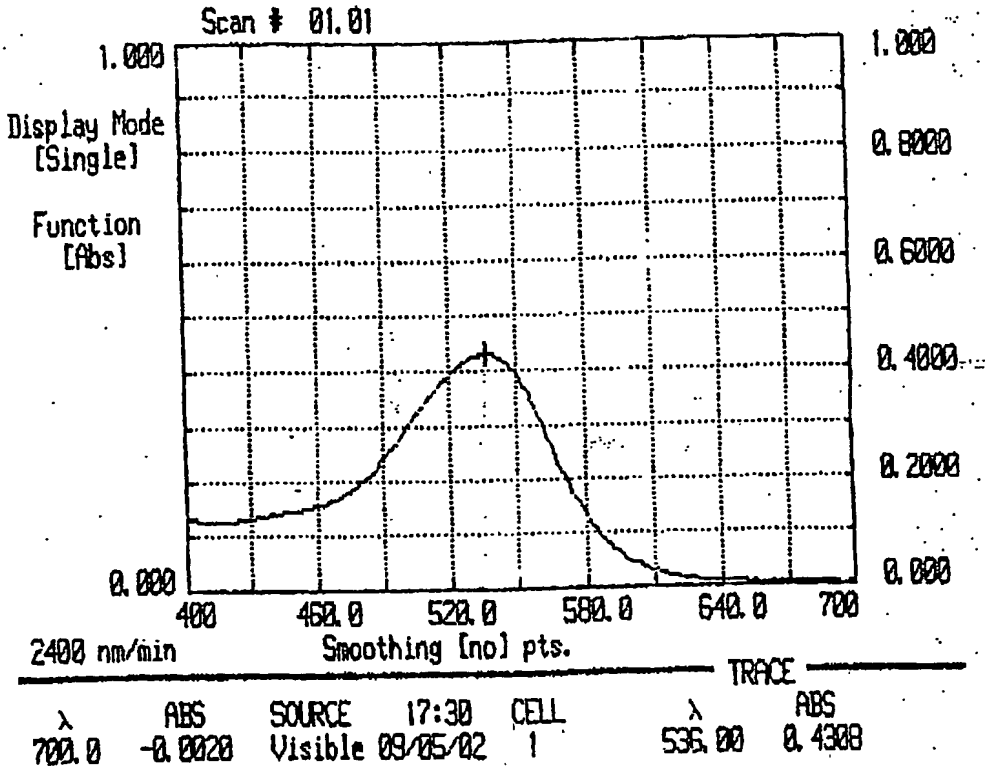


Espectro representativo do extrato de açaí, obtido a partir da amostra recebida.

Acai = Juçara

Av. Prof. Lineu Prestes, nº 580, Bloco 14 - Cidade Universitária - CEP 05508-900 - São Paulo - SP
Fone: (011) 3818-3654/3712 - Fax (011) 3815.4410

From: Alex <alex@aibmr.com>
To: Alex Schauss <alex@aibmr.com>
Date: Friday, June 14, 2002 5:44 PM
Subject: Analysis results

From: "Boxin Ou" <bou@brunswicklabs.com>
Date: Fri, 14 Jun 2002 01:24:54 -0400
To: "Alex" <alex@aibmr.com>
Subject: Re: Progress?

Alex:

Attached please find the results for Acai and Jucara extracts. Jucara contains very high level of proanthocyanidins based on our current database. Also, high antioxidant activities against hydroxyl radical and peroxynitrite.

We did not detect significant amount of phenolic compounds in Acai. I guess that the major components are fat-soluble compounds, the dark color is due to anthocyanins.

I will send you an official report which includes all the methods used in this project next week.

We will finish the anti-COX enzyme assay validation in 2 weeks, after that a manuscript will be submitted.

Regards,

boxin

[redacted]

Proanthocyanidins

Proanthocyanidins may help explain the "French Paradox," or why low coronary heart disease rates exist in French provinces known for high-fat foods and red wine consumption. Red wine could be considered an alcohol tincture of several potent flavonoids, including proanthocyanidins from grape seeds. In a provocative study, Fulvio Ursini, M.D., from the University of Padova, Italy, fed volunteers a high-fat meal with and without red wine. He found post-meal plasma peroxide levels were much lower in those who drank wine.(Ursini F, et al. Post-prandial plasma peroxides: a possible link between diet and atherosclerosis. Free Radic Biol Med 1998; 25:250-2.)

A steady stream of animal and in vitro studies supplemented by epidemiological evidence and a smattering of preliminary human studies reveal numerous health benefits associated with these compounds. Chief among the benefits is antioxidant protection against heart disease and cancer.

Proanthocyanidins--more technically oligomeric proanthocyanidins and, hence, the OPC moniker--are a class of flavonoids. Formerly called "condensed tannins," all proanthocyanidins are chemically similar, the only differences being slight changes in shape and attachments of their polyphenol rings. In nature, a jumble of different proanthocyanidins is always found together, ranging from individual units to complex molecules of many linked units (oligomers).

Proanthocyanidins are a highly specialized group of bioflavonoids that have been extensively studied since the late 1960's for their vascular wall strengthening properties and free radical scavenging activity. Proanthocyanidins are one of the most potent free radical scavengers known, possessing an antioxidant effect up to 50 times more potent then vitamin E and up to 20 times greater than vitamin C. Proanthocyanidins also have an affinity for cell membranes, providing nutritional support to reduce capillary permeability and fragility. Although bioflavonoids are widespread in nature, the powerful proanthocyanidin compound is most abundant and available from the bark of the maritime pine and grape seeds, or pips.

Bilberry extract contains anthocyanidins with claimed visual and demonstrated vascular enhancing properties. Bilberry is claimed to reduce visual fatigue and improve light to dark adjustment through its affinity for the rhodopsin-opsin system, the pigment system which mediates both light and dark vision and visual adaptation to dimly lit spaces. However, two military studies done in Israel and the United States have failed to find any such benefit from bilberry extract. The extract may, however promote the retina's own enzymatic antioxidant defenses.

In the vascular system the anthocyanidin extract supports the integrity of vascular walls by increasing vitamin C levels within cells, decreasing the permeabilizing effect of certain proteolytic/lysosomal enzymes, stabilizing cell membranes, and stimulating the synthesis of collagen and connective ground substance tissue.

Grape pips (seeds) are a potent source of proanthocyanidins, or pycnogenols. Jacques Masquelier, Ph.D., who pioneered proanthocyanidin research and coined the term "pycnogenol," used the grape seed extract in his second phase of proanthocyanidin investigations.

In vitro studies suggest OPCs also provide cancer protection. OPCs in Vaccinium-family berries, including blueberry, lingonberry and cranberry, block tumor growth by

preventing protein synthesis in tumor cells, which prevents them from multiplying.(Bomser J, and Madhavi D.L. In vitro anticancer activity of fruit extracts from Vaccinium species. Planta Med, 1996; 62:212-6.) Also in the laboratory, barley bran OPCs transformed human myeloid leukemia cells into cells that were no longer cancerous.(Tamagawa K, and Fukushima S. Proanthocyanidins from barley bran potentiate retinoic acid-induced granulocytic and sodium butyrate-induced monocytic differentiation of HL60 cells. Biosci Biotechnol Biochem, 1998; 62:1483-7.) Another in vitro study found that a patented grape seed extract killed cancer cells; inhibited growth of human breast, lung, stomach and myelogenous leukemia cells by up to 73 percent; and enhanced normal cell growth.(Ye, X. and Krohn. R.L. The cytotoxic effects of a novel IH636 grape seed proanthocyanidin extract on cultured human cancer cells. Mol Cell Biochem, 1999; 196:99-108.)

Proanthocyanidins may protect the body from a number of potentially toxic agents. Acetaminophen, the active ingredient in Tylenol, is a potent liver toxin, annually causing 75,000 cases of poisoning requiring hospitalization in the United States. Animal experiments have shown that a week of pretreatment with 100 mg/kg of a patented grape seed extract prevented liver damage from acetaminophen. Organ damage was assessed by studying liver cells for damage and also by monitoring the animal's health.(Ray SD, et al. A novel proanthocyanidin IH636 grape seed extract increases in vivo bcl-Xl expression and prevents acetaminophen-induced programmed and unprogrammed cell death in mouse liver. Arch Biochem Biophys., 1999; 369(1):42-58.)

Proanthocyanidins may do even more than prevent disease; they may make us more youthful looking. Oxidation damage causes most visible signs of aging in our skin. By preventing this damage, skin will stay younger looking. One way to achieve this is to reduce the damaging effects of ultraviolet (UV) light. Sunscreen products have incorporated a variety of antioxidants with the intent that they will prevent sun injury to the skin. In one study, grape seed OPCs exerted a solo antioxidant effect at a level of potency on a par with vitamin E--protecting different polyunsaturated fatty acids from UV light-induced lipid peroxidation.(Carini M., et al. The protection of polyunsaturated fatty acids in micellar systems against UVB-induced photo-oxidation by procyanidins from Vitis vinifera L., and the protective synergy with vitamin E. Intl J Cosmetic Sci., 1998; 20:203-15.) In this same study, the grape OPCs synergistically interacted with vitamin E, recycling the inactivated form of the vitamin into the active form and thus acting as a virtual vitamin E extender.

Part of the aging process is the degradation of skin by the enzyme elastase, which is released with the inflammatory response. OPCs specifically block elastase, thus maintaining the integrity of elastin.(Meunier MT, and Villie F. The interaction of Cupressus sempervirens L. proanthocyanidolic oligomers with elastase and elastins. J Pharm Belg., 1994;49: 453-61.)

OPCs may even help us grow a thicker head of hair, if the results of animal experiments apply to humans. Japanese researchers shaved mice and found that 40 percent of their hair grew back naturally. When a 1 percent solution of any of three proanthocyanidins was applied to the skin, however, between 70 and 80 percent of the hair grew back. Test tube studies confirm that OPCs actually stimulate the hair keratinocytes to produce three times more hair than the controls.(Takahashi T, et al. Procyanidin oligomers selectively and intensively promote proliferation of mouse hair epithelial cells in vitro and activate hair follicle growth in vivo. J Invest Dermatol.,1999;112:310-6.)

Peroxynitrite

Peroxynitrite is a cytotoxic product of nitric oxide (NO) and superoxide. Peroxynitrite is a far stronger oxidant and much more toxic than either nitric oxide or superoxide acting separately.

A variety of pathologies are associated with the formation of peroxynitrite, a potent oxidant formed from the reaction of NO with superoxide. This reaction is the fastest reaction NO is known to undergo, and transforms two relatively unreactive radicals into a more reactive oxidant, peroxynitrite. Peroxynitrite is invariably formed in larger amounts when more NO is produced, and/or when an elevated level of O2.- prevails.

Peroxynitrite is a potent oxidant implicated in a number of pathophysiological processes. Peroxynitrite freely travels across cellular lipid membranes. The calculated permeability coefficient for peroxynitrite compares well with water and is approximately 400 times greater than superoxide, hence is a significant biological effortor molecule not only because of its reactivity but also its diffusibility.(Lee, J., Marla, S.S. Peroxynitrite rapidly permeates phospholipid membranes. Proc Natl Acad Sci., 1997.)

In this regard, pathologies such as diabetes, atherosclerosis, and ischemia-reperfusion injury, are associated with oxidative stress characterized by an elevated level of O2.- that can lead to increased peroxynitrite formation. Recent evidence also suggests multiple sclerosis and Alzheimer's disease are associated with peroxynitrite formation. In addition, peroxynitrite has also been implicated during ischemia and reperfusion, and during sepsis and adult respiratory distress syndrome. Ischemia and reperfusion are accompanied by an increase in superoxide due to the activation of xanthine oxidase and NAPDH oxidase, respectively. Thus, peroxynitrite is likely to be implicated in a number of pathologies in which an imbalance of NO and O2.- occurs. [The formation of peroxynitrite is desirable for non-specific immunity but possibly not during signaling by NO.]

Peroxynitrite is formed in biology from the reaction of nitric oxide and superoxide. The enzyme Superoxide Dismutase (SOD) lowers superoxide and prevents peroxynitrite formation (see my review: Pryor, W.A. and Squadrito, G.L. (1995). Am. J. Physiol. (Lung Cell. Mol. Physiol. 12) 268, L699-L722). The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide). Peroxynitrite is a potent oxidant and itself can oxidize many biomolecules. Nevertheless, in biological systems, it reacts mostly with carbon dioxide to form reactive intermediates, such as ONOOCO2-, O2NOCO2-, CO3.-, and .NO2. Of these intermediates, only CO3.- and .NO2 participate in bimolecular reactions with biological target molecules; the CO2 adducts ONOOCO2- and O2NOCO2- are too short-lived and decompose before they can react bimolecularly.

Oxidative stress, such as that caused by peroxynitrite is known to damage the vascular endothelium, a process that can lead to atherosclerosis (Thom, S.R. and Ischiropoulos, H. Mechanism of oxidative stress from low levels of carbon monoxide. Health Effects Institute Research Report, number 80, 1997.)

Hydroxyl Radical

If the function of radicals is to destroy molecules and tissues, then the hydroxyl radical would be the radical's radical. It reacts at diffusion rates with virtually any molecule found in its path including macromolecules such as DNA, membrane lipids, proteins, and carbohydrates. In terms of DNA, the hydroxyl radical can induce strand breaks as well as chemical changes in the deoxyribose and in the purine and pyrirnidine bases. "

"Damaged proteins, many of them crucial enzymes in neurons, lose their efficiency and cellular function wanes. Protein oxidation in many tissues, including the brain, has been proposed as an explanation for the functional deficits associated with aging.

The hydroxyl radical is a third generation species of radical which is derived from hydrogen peroxide (HZ02), which, in turn, is derived from the superoxide radical through the action of the enzyme superoxide dismutase.

Hydrogen peroxide is reduced to hydroxyl radicals by the enzymes glutathione peroxidase and catalase in the presence of transition metals such iron or copper.

Phenolic Compounds

Luteolin:
Inhibits proinflammatory cytokine production in macrophages. An anti-cancer flavonoid: poisons eukaryotic DNA topoisomerase I.

Note: There is an entire section in an annual nutrition review journal about to be published on the safety, metabolism of bioavailability of dietary flavanoids within a few weeks.

[redacted]

I. Characterization of Anthocyanins

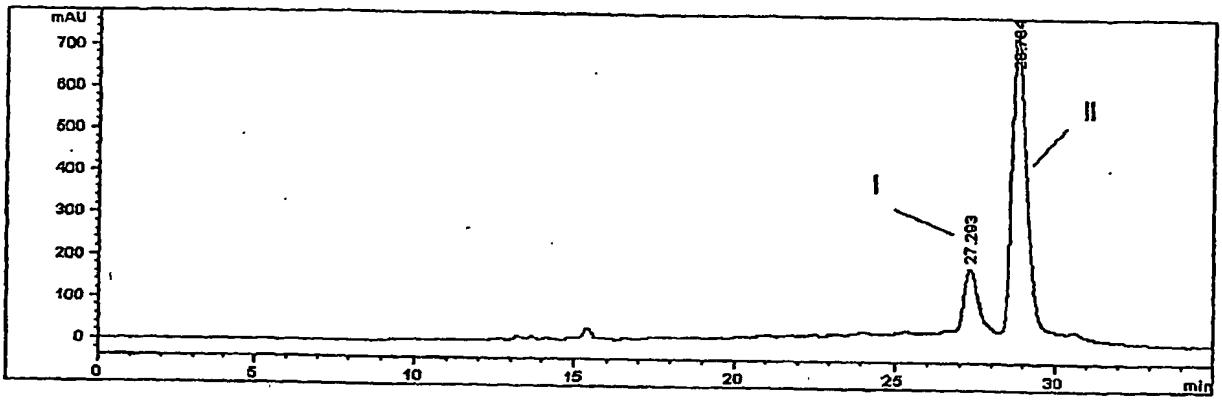
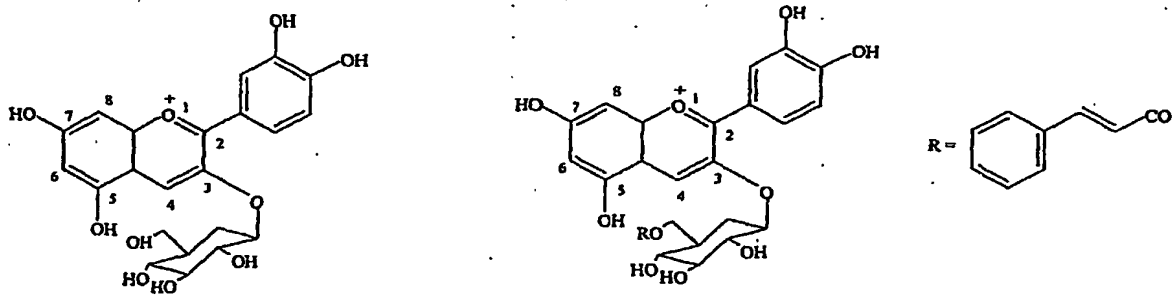


Figure 1. Anthocyanins Profile of Jucara Extract

Table 1. LC/MS/MS results for peaks shown in Figure 1

Peak	Retention Time	Molecular Ion	Product Ion
I	27.29	449	287
II	28.78	595	449,287



I: Cyanidin-3-glucoside II: Cyanidin-3-glucoside-coumarate

Figure 2. Structures of Anthocyanins from Juraca

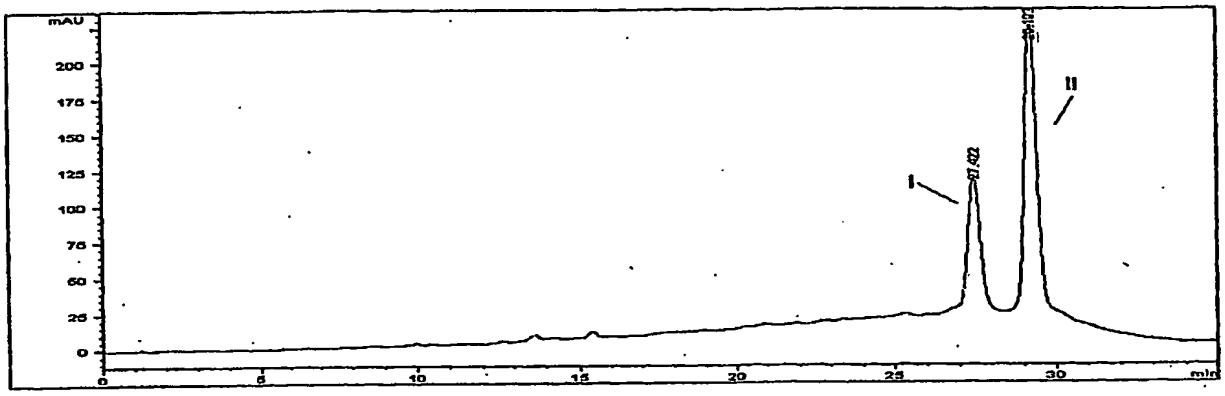
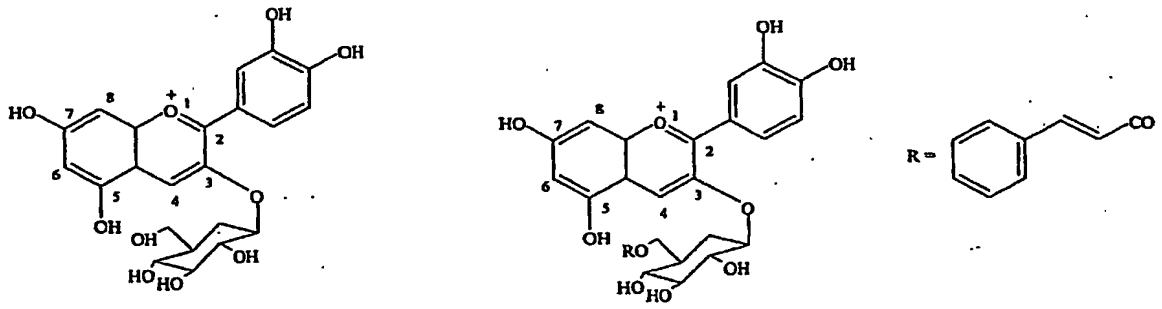


Figure 3. Anthocyanins Profile of Acai Extract

Table 2. LC/MS/MS results for peaks shown in Figure 3

Peak	Retention Time	Molecular Ion	Product Ion
I	27.42	449	287
II	29.19	595	449,287



I: Cyanidin-3-glucoside II: Cyanidin-3-glucoside-coumarate

Figure 4. Structures of Anthocyanins from Acai

Table 3. Contents of Anthocyanins

Anthocyanin (mg/g)	Jucara	Acai
Cyanidin-3-glucoside	3.43	1.77
Cyanidin-3-glucoside-coumarate	17.56	3.93
Total	20.99	5.7

II. Characterization of Individual Phenolics

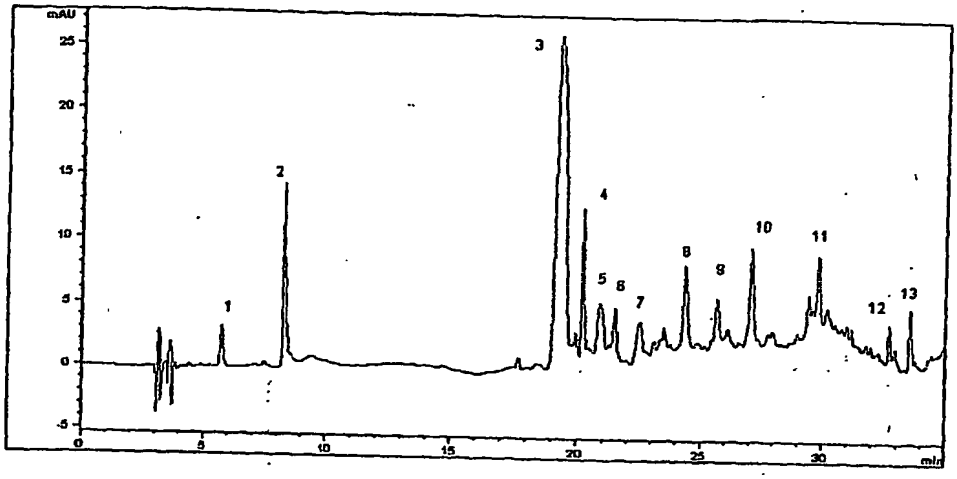
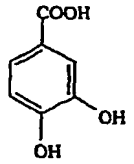


Figure 5. HPLC Profile for Individual Phenolic from Jucara Extract

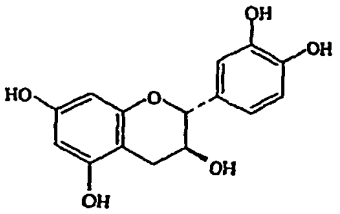
Table 4. Identity of Phenolic Peak and its Mass Spec Data

Peak	Retention Time	*[M-1]	**MW	Structure
1	5.72	153	154	Protocatechuic acid
2	8.23	289	290	Catechin
3	19.24	449	450	Eriodictyol-7-glucoside
4	20.17	447	448	Luteolin-4'-glucoside
5	20.84	463	464	Isoquercitrin
6	21.64	547	548	Unknown
7	22.46	433	434	Quercetin-3-arabinoside
8	24.32	287	288	Eriodictyol
9	25.63	249	250	unknown
10	27.02	285	286	Luteolin
11	29.77	299	300	Chrysoeriol
12	32.66	343	344	Eupatorin
13	33.49	285	286	Kaempferol

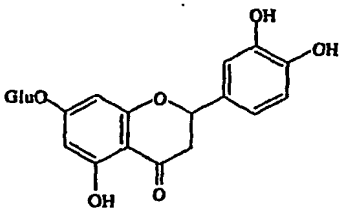
* molecular ion (negative mode)
** molecular weight



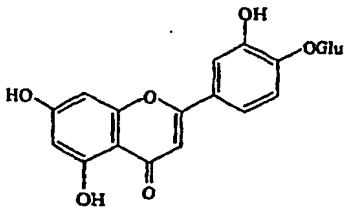
protocatechuic acid



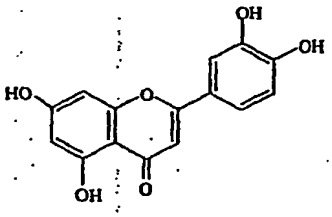
catechin



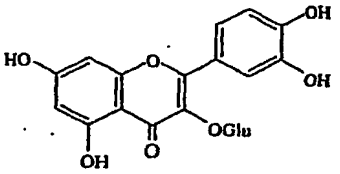
Eriodictyol-7-glucoside



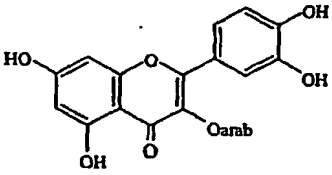
Luteolin-4'-glucoside



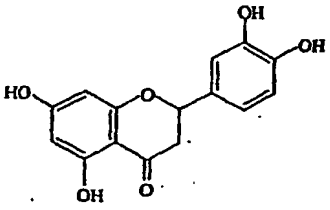
Luteolin



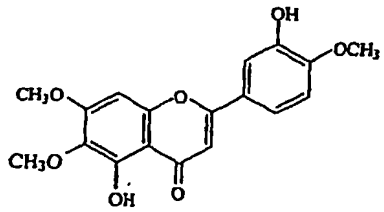
Isoquercitrin



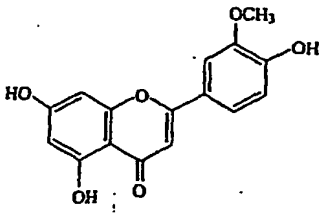
Quercetin-3-arabinoside



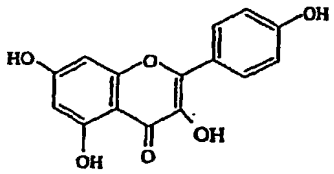
Eriodictyol



Eupatorin



Chrysoeriol



Kaempferol

Figure 6. Structures of Identified Phenolics

III. Quantitation of Proanthocyanidins

Table 5. The content of procyanidins in freeze-dried samples, (mg/g, mean ± SD, n=3)

Proanthocyanidins	Jucara	Acai
Monomers	0.35	0.21
Dimers	0.52	0.30
Trimers	0.29	0.25
Tetramers	0.87	0.32
Pentamers	0.50	0.31
Hexamers	1.03	0.52
Heptamers	0.60	0.32
Octamers	0.72	0.39
Nonamers	1.40	0.64
Decamers	0.55	0.34
Polymers	18.53	9.28
Total	25.38	12.89

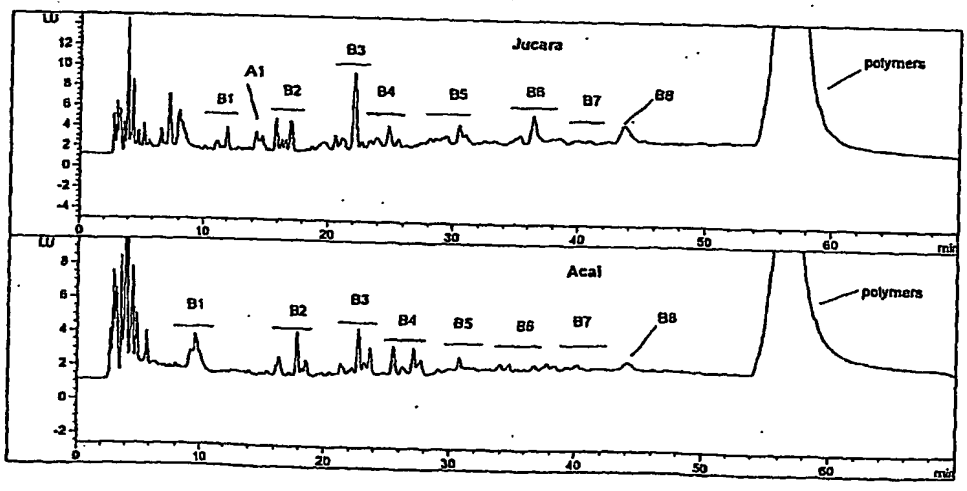
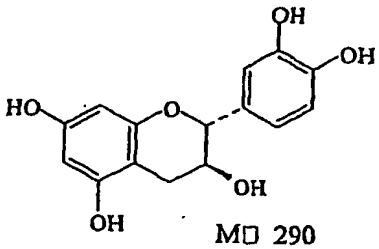
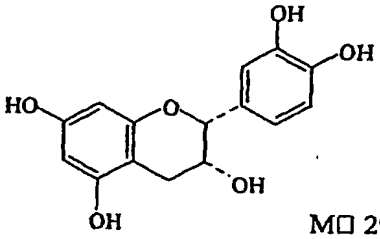


Figure 7. The profile of proanthocyanins. B1 are epicatechin and catechin. Peaks B2 through B8 stand for the B type procyanidin from dimers to octamers. A2 are dimers with one A type inter-flavan linkage as reflected by the mass spectra.



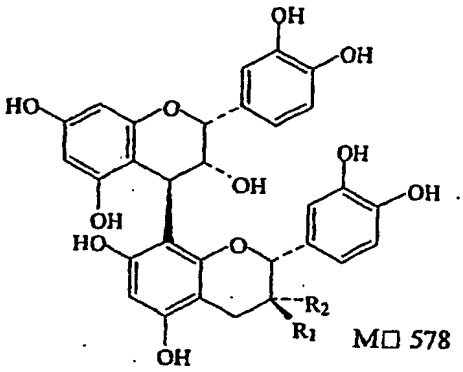
M□ 290

1. catechin



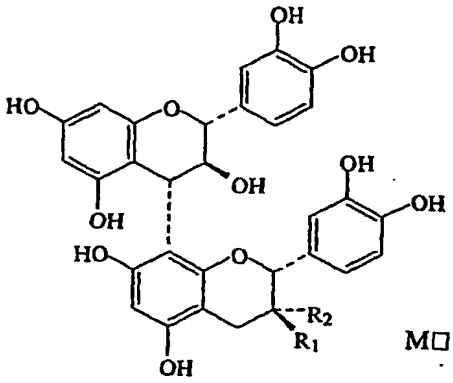
M□ 290

2. epi-catechin



M□ 578

3. R1= OH, R2= H; B-type dimer 1
4. R1= H, R2= OH; B-type dimer 2



M□ 578

5. R1= OH, R2= H; B-type dimer 3
6. R1=H, R2= OH; B-type dimer 4

Figure 8. Representative Structures of Proanthocyanidins

IV Measurement of Antioxidant Activities Against Hydroxyl Radical and Peroxynitrile

Samples	HORAC	NORAC
Jucara	85	134
Acai	52	34

Note: a. The HORAC result is expressed as microl mole gallic acid equivalents per gram

b. The NORAC result is expressed as microl mole Trolox equivalents per gram

BRUNSWICK LABORATORIES
6 Thacher Lane • Wareham, MA 02571 • USA
Telephone 508-291-1830 Fascimile 508-295-6615

Report for American Institute for Biosocial and Medical Research, Inc.

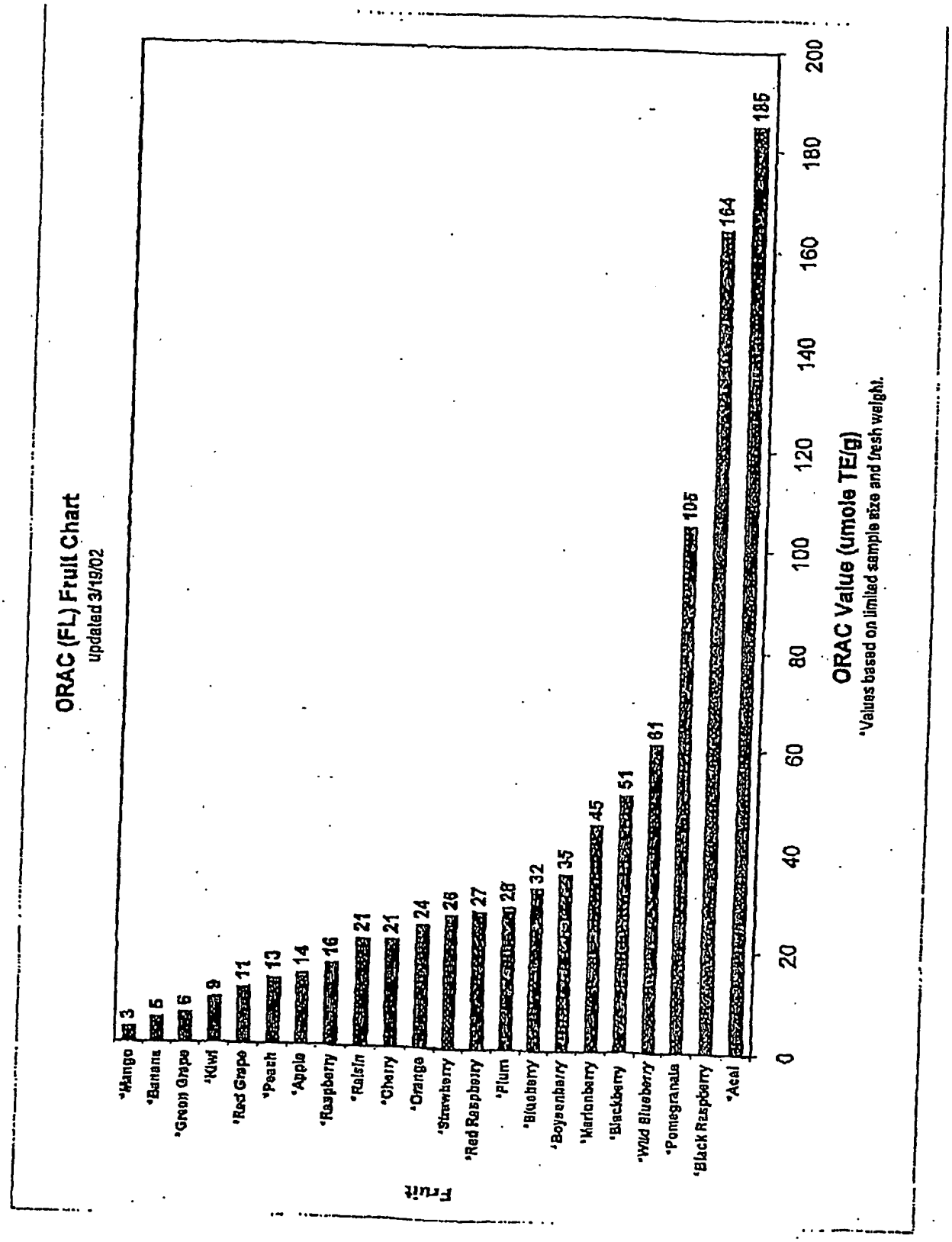
Sample ID	Brunswick Lab ID	$ORAC_{FL}$* (µmoleTE/g)
Jucara fruit pulp Freeze Dried Lot#2204	02-487	1,193

*The $ORAC_{FL}$ analysis, which utilizes Fluorescein as the fluorescent probe, provides a measure of the scavenging capacity of antioxidants against the peroxyl radical, which is one of the most common reactive oxygen species (ROS) found in the body.

Trolox, a water-soluble Vitamin E analog, is used as the calibration standard and the ORAC result is expressed as micromole Trolox equivalent (TE) per gram.

Testing performed by J. Flanagan.

B-915 / 5-14-02


ORAC (FL) Fruit Chart
updated 3/19/02
*Values based on limited sample size and fresh weight.
ORAC Value (umole TE/g)
0
20
40
60
80
100
120
140
160
180
200
Fruit
*Mango 3
*Banana 5
*Green Grape 6
*Kiwi 9
*Red Grape 11
*Peach 13
*Apple 14
*Raspberry 16
*Raisin 21
*Cherry 21
*Orange 24
*Strawberry 26
*Red Raspberry 27
*Plum 28
*Blueberry 32
*Boysenberry 35
*Marionberry 45
*Blackberry 51
*Wild Blueberry 61
*Pomegranate 105
*Black Raspberry 164
*Acai 186

A ai

Biochemical and Technical Studies on A ai

(Euterpe Oleracea, Mart)

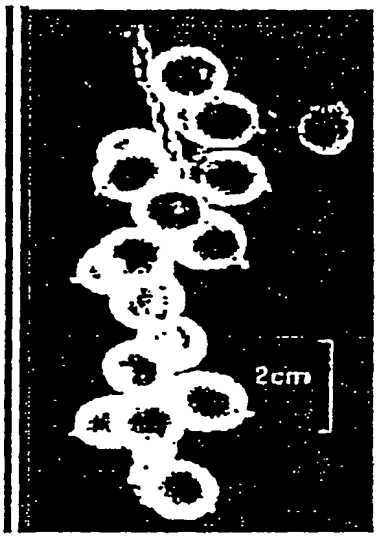
Summary excerpted from a study by
Dr. Herv Rogez [1,2], Sophie Pascal [2],
Jesus N.S. de Souza, Arlete R. Aquino & Raphaele Buxant[1,2]
[1] Dépt. de Engenharia Quimica - Centro Tecnol gico, University of Par , Bel m, Par , Brasil
[2] Unidade de Bioquimica da Nutri o - Universida Cat lica de Louvain, B lgica

Microbiological and Biochemical characteristics.

2. General Considerations

The palm is cespitose, that is, it grows in clumps of trees/roots formed by successive burgeoning that come from only one dispersion unit (each root can reach 20ft). Above the trunk (estipe), the inflorescence burgeon. Each one originates a cluster of fruit (hundreds of fruits). The palm fruit has a round-like shape and its size is from 1 to 1.5cm. The bunch average weight changes according to season, species and age of the plant; its weight can reach 4-5 Kg (1-2 Kg of fruit) (Bovi and Castro, 1993). Picture 1 shows a piece of a bunch with several fruits and a seed (right side). From this picture, it s possible to notice that the seed is responsible for the greater volume of the fruit. And the proportion of the pulp accounts for only 10 to 20%, according to species and age of the fruit. A detailed analysis of this pulp will be shown in the next chapter.

The seed (endocarp and nut) can be used as organic material, providing an excellent fertilizer for the cultivation of vegetables and ornamental plants. The nut produces green oil, used at traditional medicine as an anti-scrofulous (Bovi and Castro, 1993)



PICTURE 1 - Piece of a bunch with 16 fruits (left) and a seed (right) of A ai (*Euterpe oleracea* Mart.) (*Cavalcante*, 1991)

The palm bears fruit only after the third year, reaching its maximum production in the 5th and 6th year. There are two harvest times (Calzavara. 19972). The Winter harvest is from January to June: the quantity of fruit found in the market is low and the fruits are in different maturation conditions in the same bunch, which contributes to the devaluation of the product (A ai juice of different tastes). Summer harvest is from August to December (reaching its maximum production between August and November); the fruits are numerous, and their maturation is uniform and the taste of the juice is better.

2.2.Composition of pulp and A ai juice

The two following tables show the summary of the literature about the chemical compositions of the pulp and of the A ai juice. Every result shown in table 1 was converted in order to express the

importance of the macro- and micro- nutrients per gram of dry material and make it able for you to compare the data among them. Table 2 shows the fatty acid profile found in the lipase.

TABLE 1: Chemical composition in the pulp and A ai juice and energetic values

(gm = dry material gram)

Component	Costa (1936) pulp	Motta (1946) pulp	Chaves (1948) pulp	INCAP (1961) pulp	Rocha (1966) pulp	Endef (1977) pulp	Motta (1946) juice	Chaves (1948) juice	Lehti (1993) juice	Roge (199 juice
pH	–	–	–	–	5.9	–	–	–	–	5.8
Energy	111.7	–	80	265	–	247	–	–	–	–
Water	38.6	59.73	41.0	41.0	50.88	45.9	87	85	85	85
Dry Material	61.4	40.37	59.0	59.0	49.12	54.1	13.0	15	15	15
Protein	5.70	6.24	5.73	5.76	9.65	7.02	18.23	8.33	–	13
Total lipids	17.92	17.34	22.71	20.68	24.75	22.55	45.85	50.67	–	48
Total sugars	.65	63.22	20.37	20.68	24.49	36.42	–	6.67	–	1.5
Total reducers	–	–	–	–	19.42	–	–	–	–	1.5
Fructose	–	–	–	–	–	–	–	–	–	0
Glucose	–	–	–	–	–	–	–	–	–	1.5
Sucrose	–	–	–	–	4.82	–	–	–	–	0
Fiber(cellulose)	74.59	–	30.51	30.51	32.37	31.24	32.30	32.33	–	34.0
Ashes	1.14	3.05	2.12	2.03	3.58	2.77	3.62	2	–	3.5
Micro sodium	–	–	–	–	–	–	–	–	16	56.4
Macro Potassium	–	–	–	–	–	–	–	–	499	932
Macro Calcium	–	414 (CaO)	–	–	–	218	385 (CaO)	187 (CaO)	–	286
Macro Magnesium	–	–	–	–	–	–	–	–	121	174
Micro Iron	–	10 (Fe_2O_3)	–	–	–	21.8	7 (Fe_2O_3	14.7 (Fe_2O_3)	26(?)	1.5
Micro Copper	–	–	–	–	–	–	–	–	2	1.7
Micro Zinc	–	–	–	–	–	–	–	–	2	7.0
Sulfur	–	–	–	–	–	–	–	367 (SO_3)	–	–
Macro Phosphorus	–	126 P_2O_5	–	–	–	107	254 P_2O_5	227 P_2O_5	–	124
Vitamin C	–	–	–	–	–	17	–	–	–	–
Thiamin (B_1)	–	–	–	–	–	0.67	–	–	–	0.25
Riboflavin (B_2)	–	–	–	–	0.2	0.02	–	–	–	–
Niacin (B_3)	–	–	–	–	0.8	0.7	–	–	–	–
Vitamin E	–	–	–	–	–	–	–	–	–	45
TOTAL	–	89.85	81.44	79.66	–	–	–	–	–	–

TABLE 2: A ai juice oil composition according to two different works.

Fatty Acids	Lubrano 1994 Oil	Rogez 1996 Oil
C16:0 (%)	22	23
C16:1 (%)	2	4
C18:0 (%)	2	1
C18:1 (%)	60	50
C18:2 (%)	12	12
C18:3 (%)	Some	1
C20:0 (%)	2.5	–

After studying these two tables above, we can understand the following:

- There is a significant difference between the pulp composition and that of the juice. The lipase content is greater in the juice than in the pulp and on the contrary, the sugar content is lower. The mechanical hull of the pulper (*cfr.* Unit 4 and Picture 3) includes a filtration through a thin sieve (very small holes: 0.4mm diameter). Therefore, part of the fibers and the polysaccharide of the pulp doesn t pass through it like the lipase, that is less rigid. As the juice, not the pulp, is usually consumed, you may consider only the juice composition for the following nutritional discussion.

The A ai fruit is an important base food. The quantity of lipase (about 50% of the dry material) guarantees you an energetic value twice as high as milk. The oil extracted from the A ai is a good quality fatty acid, with 60% of mono-unsaturated and 13% of polyunsaturated, a composition that is very similar to the *Elaeis oleifera* palm oil, that is also from the Amazon region (Lubrano *et al.*, 1994). This fat is, therefore, better for health than that of the milk (highly saturated animal fat) and it is possible to notice the presence of a fat-soluble E vitamin (6.8mg/100g of juice, that is 45mg/100g of dry material. The E vitamin (tocopherol) has a very important natural antioxidant role and can act in the elimination of free radicals, (de Luca and Suttie, 1969). The daily quantity of E vitamin needed is 5-15 mg.

The fiber content (the main component is the cellulose, a glucose-based polysaccharide) is high (about 32-34% of the dry material), what is very favorable to the consumers proper intestinal process. Even though the fibers aren t absorbed in the intestine, it s known that they accelerate the intestine process and that they have a very important role in avoiding colon cancer . The D.V. (Daily Values) is 35g of fibers/adult a day. A ai consumers reach this dose very easily (with only one dose of half a liter of a medium-size A ai, the consumer will be consuming 25g of fibers).

The total sugar content is relatively low in the juice (1.5 —6.67%). The detailed study of these sugars made by liquid chromatography (Rogez *ET al.*, 1996) shows only the presence of glucose, a reducing sugar (absence of fructose and sucrose).

It s possible to notice a reasonable protein content (8-18% of the dry fruit. However, that content isn t enough to supply the protein needs, especially for kids and adolescents (growing phase). Children must have other protein source in their alimentation.

A ai can be considered one of the richest foods in iron (1.5-5 mg/100gs), that s why this nutritional aspect has always been covered. However, that doesn t mean that it is available to be easily absorbed by the intestine. The iron absorption depends on the way it is presented in the products. In general, the human being absorbs only 5% of the vegetable iron, where it s found in free form, and 25% of the animal iron, where it s essentially linked to hemes groups, which eases its transportation in the intestinal barrier. Besides, in botanicals, the iron bioavailability is influenced, either in a positive or in a negative way, by the presence of other components of the plant. For example, the C vitamin aids absorption by kelating the iron (x 3-6 times), however there is only a little C vitamin naturally available in the A ai. Additionally, the tannins and other polyphenolic components, cause the formation of iron tannin, insoluble in the intestine and not available to the absorption. That s the A ai juice case.

The potassium and especially the calcium contents (43 mg/100g of A ai juice compared to 120 mg/100ml in the milk) are high and contribute to make this product a reasonably complete food.

Also present is a small amount of vitamin B1 (0.25 mg/100gs). The DV for the adults is 1.3-1.5 mg, depending on the sex (500 grams of Acai would yield 1.25mg). The lack of vitamin B1 can cause beriberi. The B1 vitamin is thermal resistance and is kept in the processed A ai juice, in spite of the several thermal treatments performed (points 3 to 7)

The A ai juice has as purple-like color. When lemon juice or other acid substance is added to the juice, allowing the juice pH to be lowered, it becomes red. If the pH increases (addition of soap or other substance containing basic pH), the juice will become blue. This change in color caused by the pH is due to a particularly high content of anthocyanin pigments: 336 mg/100g of juice (Iaderoza, 1992). This value can be compared to of red wine (about 100 mg/liter of wine). Anthocyanins are polyphenolic structure pigments that have an antioxidant effect, protecting against the production of atheroma plaques, and play a very important role in the vascular cardiac system, favoring a better blood circulation.

A ai is considered a new potential source of natural red coloring matter for use.

Nothing has been found in the composition of the A ai that would cause any reaction when mixed with other food. The A ai can be mixed with citric juices, beer, milk, etc. Hence, there is nothing against the consumption of these foods simultaneously in reasonable quantities.

In summary, the A ai juice is a highly caloric drink (due to the high lipase tenor), with high fiber tenor, anthocyanin and other micro-nutrients. Therefore, it can be considered one of the most nutritional fruits from Amazon.

2.3. Enzymes: peroxides, lipoxigenase and polifenoloxidase

The main cause of the degradation of the A ai juice are enzymes, not only those produced by the contaminating microorganisms but also those originated from the fruit.

For example, based on the lipase level, two main enzymes predominate (Deroanne and Bouffioux, 1984).

- the lipase degrades the glycerol triacid into free fatty acid.
- The lipoxigenase acts on the polyunsaturated free fatty acids (linoleic and linolenic acids) and produces some degradation products, changing the taste of the juice. Besides, co-oxidation phenomenon leads to the degradation of the polyphenols, changing also the color of the food.

The darkening, called enzymatic, of the juice may be caused by the action of two enzymes: the poliphenoloxidase and the peroxidase that are responsible for the appearance of brownish polymers . (V mos-Vigy so, 1981). Peroxidase shows high resistance to heat (it s the most thermal resistant of all). That s why it is used as an indicator in the evaluation of the thermal treatments applied to the juice in order to preserve it. Therefore, if the peroxidase is totally inactive after processing, the process used will also destroy the other enzymes.

In research it s important to notice if these enzymes are active in the A ai juice, their respective activities should be measured to determine whether or not they are high and which has the most negative impact in the juice. This data will make it possible to know if it s necessary to employ a preemptive chemical or thermal inhibition of the enzymes on a technologic basis. Protocols are being elaborated for determining the enzymatic activities of the peroxidase and the polifenoloxidase by spectrophotometry, making them visible like the enzymes of the lipoxigenase by polarography . This work is undertaken with the help of the Nutrition Biochemical Unit from Catholic University de Louvain.

2.4 Microbiology

2.4.1 On a quantitative basis

One of the factors that makes the A ai perishable is the high microbial load present in the fruit (Oliveira, 1988). A ai has a large surface area of contact with the environment, if compared to its pulp weight, and it has a composition (rich and with low acidity) that favors the growing of microorganisms.

Veloso (1994) studied the occurrence of several microorganism of great importance for the food technology. The total load of A ai (per gram of dry material) has a value between 10^7 and 10^9 microorganism (from 1 to 10% of mold and leaven/yeast). The total value of colliform is very high (in each sample, it was found over 1100bac/g of juice). The presence of fecal Colliforms, Salmonella and Staphylococcus aureus was detected in about 10-20% of the samples.

2.4.2 On a qualitative basis: Identification of the main molds and leavens

As the microbial load of the A ai is very high, an additional study was performed at the mycology lab of the Catholic University of Louvain (Belgium) in order to identify the main molds and leavens. The study shows the identification of the mold and leaven contaminating agents extracted from samples of fresh A ai (*Euterpe oleracea*) (fruits, juice, thrash), from several origins, aiming at a better understanding of the difficulties found in the conservation of the A ai products.

The following factors can explain the A ai high microbial load:

- the substract is good for the growing of the contaminating agents (neither acid nor sweet)
- the ratio between the A ai surface in contact with air and the pulp weight is considerable.
- the A ai palm grows in humid and hot places, what are favorable to the growing of microorganism and insects
- The lack of care during the harvest and the transport of the fruit are the origin of supplementary contamination by contact with contaminated surfaces (soil, plastic, and containers).

Over 20 kinds of mold, filamentous fungus were identified: *Aspergillus, Penicillium, cladosporium, Fusarium, Trichoderma, Coilomyc tes* (*phoma, phomopsis, geotrichum, coniella*), *Mucorales and Hyphomycetes* (*trichospron, sporothrix, trichosporiella*) Several *Aspergillus* and specially the *Penicillium* are potential producers of mycotoxin. Most of the molds accept a water activity up to 0.75 (more resistant than the bacteria). They usually have low resistance to heat, except some identified species whose structures of resistance (ascospores, chlamidospores or sclerotes) need a temperature of 80...C for some minutes for their elimination.

The leaven identification is based on the knowledge of the assimilation profiles and the fermentation of the heather(cepa), i.e. the assimilation and fermentation of sugars and alcohol, the need for nitrogen substances and vitamins, aerobics or anaerobic). The A ai isolated leaven population indicates an assimilation profile generally very large (typical of a varied substract, that isn t specifically sweet.). Half of them have fermentation capacity. Those leavens often originate from soil (for example, the species of the *Cryptococcus*) and heather commonly found in several *substracts*. Others are found in the leaves (such as the *Sporobolomyces, Thodotorula and Cryptococcus*) or in the fruits. Intermediaries of insects (such as the *Hanseniaspora uvarum*) bring some of them. The most frequently found heather is the *Debaryomyces hansenii*.

Those leavens have different characteristics for heat resistance (numerous ascogenas leavens), antibiotics, weak water activity (several osmofilas leavens), pH and in presence of conservatives. Common aspects were noticed among the assimilation spectrums of the undetermined heather (probably due to genetic variability and to adaptation capacity).

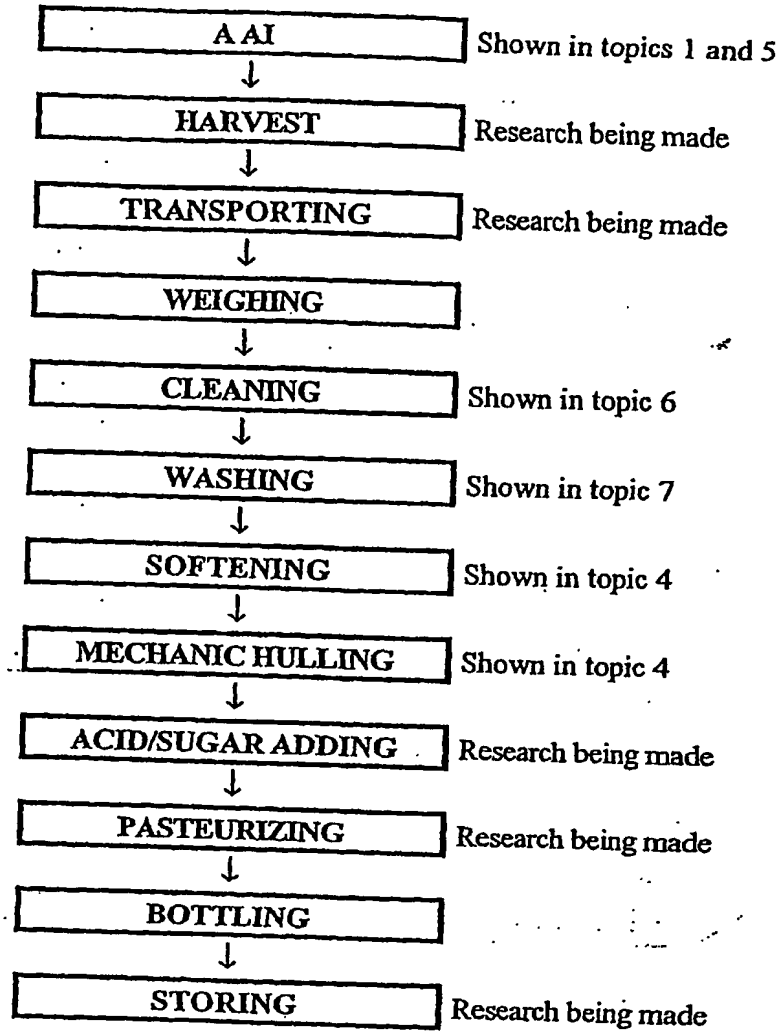
Not only a quantitative but also a qualitative difference is noticed among the A ai samples that come from several regions. The microbial flora diversity varies according to its precedence.

3. Research Uncertainty

The Federal University of Par (Chemical Engineering Department) and the Catholic University of Louvain (Nutrition Biochemical Unity, Belgium) assist, through a research agreement covering the A ai juice characteristics and the ways it can preserved by using technology available in the rural communities. Those researches focus on two main aspects:

1. The best knowledge about the A ai juice and fruit itself concerns its **biochemical and microbiology.** They tried to determine the contamination origin of the fruit, changes in its color, the influence of the temperature and weather on its conservation, evolution of its nutritional and microbiological characteristics as time goes by.
2. They tried to improve juice conservation, mainly through the **reduction of its microbial load,** by performing light treatments, which allow the nutritional and organoleptical characteristics to be preserved.

In the long term, it s hoped that the conservation of the juice, after bottled, will last several months in room temperature, with the use of suitable treatments. Significant advances related to the knowledge of the fruit and its degradation processes (change of its quality year by year and during its ripening process) are expected. Understanding those processes means being able to improve conservation and quality of fresh fruits after its harvest.



Picture 2 - Diagram of the A ai juice preparation, including washing of the fruits and their pasteurization.

A better conservation of the fruit and of the juice will allow consumption of the food while preserving its nutritional value and will ease the organization of its commercialization, between harvest . The preparation and processing steps of the A ai juice are shown in Picture 2. The researches carried out deal with the optimization of each steps of that processing.

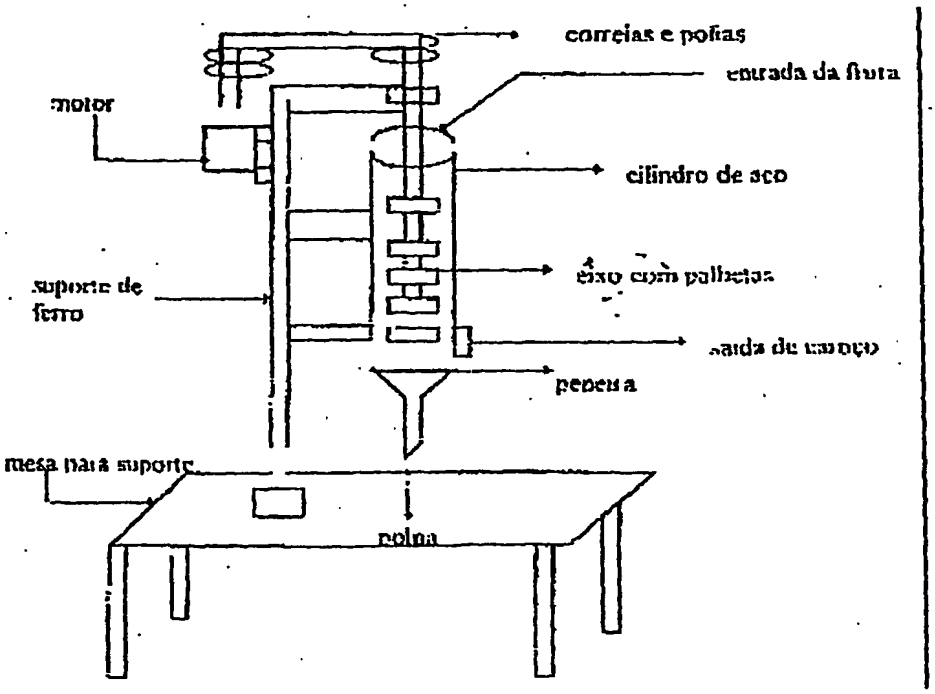
In Picture 2, the main topics of the studies developed with this product are illistrated. The hulling of the fruit can be done in several different ways and the softening conditions change from one producer to

the other. That s why a research about that changing aspect was done (topic 4. Optimization of the pulp extraction process). As the contamination of this fruit is very high, the evolution of the microbial load was studied according to the post-harvest time (topic 5. Evolution of the microbiological characteristics of the A ai fruits in the post-harvest). At a later phase, several treatments were applied on the fruit before its hulling (topic 6. Cleaning, and topic 7. Washing of fruits).

4. Optimization of the pulp extraction process — softening and mechanical hulling.

Fruit hulling can be performed in several ways, each producer has its own way for processing the A ai (softening time and temperature, amount of water per kg of fruits, time that the fruit stays in the machine). So, in order to maintain reproducibility among our experiences and to assure that we had a good output, it was necessary to optimize and systematize the pulp extraction process.

Picture 3 — A ai hulling



The A ai fruit hulling takes place, most of the time, in a mechanical hulling with vertical axis, specifically elaborated and used for the A ai fruit. The picture of the mechanical hulling is shown in Picture 3.

It was designed for processing 2kg of A ai each time, in order to minimize the amount of fruits in each processing.

During interviews made with the A ai producers and fair-trader who extract the juice from the fruit in Bel m, the harvest conditions of the fruits, ways for preparing A ai and problems found concerning transport and sales were analyzed.

1. It was noted that the time and temperature of water for soaking the fruit change a lot from one fair-trader to the other. Each one has its own preference: some of them say that it s better soaking in running water at room temperature for some hours before beating, others say that it s better softening the fruit in warm water for a short time (10-20 minutes). According to the interviews made, it s likely that the origin of the fruit is significant for those differences in soaking times and temperatures.

In the laboratory, several softening times (0,5,10,15,20,30 minutes) and soaking water temperatures were tested (30,40,45,50 and 60...C)The results show that there are no significant differences between the different conditions of softening, it was noticed that there was a tendency for a greater output for the parameters 20 minutes under water temperature of 45...C .

2. The hulling total time, as well as the amount of water and the way it s used during this time, constitute very important variables to the output and density of the juice. Five total times of hulling were tested (altering from 2 30 to 5 00) and for each one, several hulling times were tested before putting the first dose of water, and several ways for putting the water inside the hulling machine (the time for the dripping of the juice at the end of the hulling was set to 45 seconds). The total amount of water was 1 liter per 2 Kg of fruits (in order to get an ideal juice; not very strong, not very weak).

From these researches, it was noticed that the total time for hulling and the way of putting water inside the hulling machine have a very significant effect on the outputs in dry substances. From those experiences, the following protocol for the preparation of the juice was set:

1. Weigh 2kg of raw material(A ai)
2. Prepare 5 containers with 200ml of water each
3. Place the A ai in the machine, turning it on at 0 time
4. After one minute of hulling, put the first 200ml water container at once
5. At the times 1 30 , 2 00 , 2 30 and 3 00 , put the other four containers left the same way
6. Leave it dripping for 45 seconds until it reaches the 3 45 time

The recycling impact of part of the juice was analyzed. Although this technique for preparing the juice is very popular, no differences in the outputs of dry substances were noticed. This technique is supposed to be used by the fair dealers in order to shorten the hulling time.

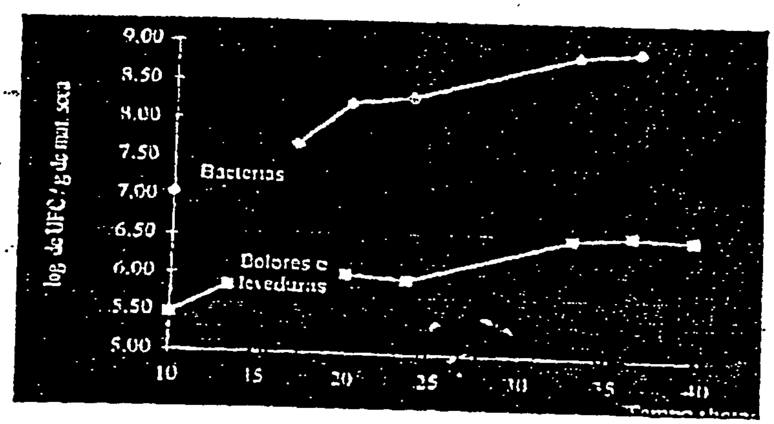
5. Evolution of the microbiological characteristics of A ai fruit after its harvest.

When A ai characteristics and qualities are compared at harvest and at middle harvest, significant changes were noticed, in the organoleptic qualities and in the numbers of the scientific indicators (microbiological, dry weight, color).

- The A ai of the harvest sold in Bel m, is cheap and abundant. It has good quality, because as it comes from places near Bel m, and it doesn t suffer changes during transportation.
- On the other hand, between harvests, A ai is produced at lower quantities, its organoleptic characteristics are inferior to those of the A ai from harvest time and it s more expensive. It comes from more distant places (Maranh o, Maraj island), and undergoes a long trip before reaching Bel m s harbor. Time between harvest and sale/consumption of fruit is very important (up to 48 hours, what is almost the maximum time the fruit lasts after harvest at room temperature).

An increase at the A ai microbial load between_harvest was noticed, when compared to that from harvest time (during harvest time, average load of fresh A a of 10^6 microorganism per gram of dry pulp — what accounts for 10 ml of juice); between harvest, that load is of 10^9, which means that thepresence of a microbial load is 1000 times higher).

It was necessary to determine if the contamination increase was caused mainly because of low quality of the pàlms of those regions, poor transport conditions, or because of the increase in the time after harvest, which would lead to natural growth of the microorganisms already present at the fruit surface. Influence of time spent between harvest and processing was, then, related to the increase in the microbial load. The results are shown in Picture 4.



Picture 4 —Increase of microbial load over time.

During the experienced time (30 hours from first sample, the one that was taken 10 hours after harvest), there s a noticeable and regular increase in the original microbial load of the fruit.

The microbial load is about 10^5-10^6 microorganism (bacteria, mold and leavens) per gram of dry pulp right after harvest and after 40 hours reaches a maximum value, a little superior to 10^9 microorganism per gram of dry pulp. It was noticed, 40 hours after harvest, that the A ai has a microorganism load similar to that of the middle harvest. Consequently, it s possible to conclude that the microbial load variation in the harvest time and out of it is mainly caused by natural increase of microorganism on the surface of the fruit.

Thus, A ai should be used right after its harvest, before a significant increase of microbial load happens, what would bring an alteration to the product (not only a natural change caused by the microorganism

increase but also a change caused by necessity of using thermal radical treatment in order to preserv the product).

6. Cleaning

The efficiency of cleaning methods on the decrease of microbial load of juice was studied. The studie were made using the middle harvest A ai, which means A ai that has an important level o contamination. The cleaning of the fruit with hygienic water at 0.1%(v/v) concentration, befor processing, allowed the microbial load to be reduced from 2 to 400 times (concerning the A ai cleane with potable water without addition of chemical substances). Such concentration doesn t cause an organoleptic change in the juice. However, this significant decrease, when analyzing an A ai with a higl original microbial load (from the middle harvest), becomes insignificant when cleaning is performed in a fresh A ai with low original contamination (from harvest time).

1. Washing

Washing consists of a not very severe treatment and it s taken with the objective of decreasing the microbial load of the product before the transformation. In the case of the A ai fruit, that will allow it to consequent treatments, aiming at the conservation of the juice, and therefore, the preservation of its organoleptic, texture and nutritional qualities. (Tournas, 1994)

Washing consists of placing the fruits in hot or boiling water or steaming for some time before processing (Cruess, 1995). The choice of that treatment, aimed at decreasing the contaminating agents present on the surface of the fruit, is explained by the physical structure of the fruit (Unit 1.2). The fruit that has only one small layer of superficial pulp, a short contact time between the pulp and the hot water or steam leads to a positive efficiency of the treatment at not very high temperature or long time.

Researches were made, not only with the A ai from harvest time but also those from middle harvest, trying at several different temperature (from 75...C to 100...C) and several washing times (from 5 seconds

to 10 minutes) (Rogez *et alii*,1996). Each of them had a significant impact over the reduction of the microbial load (bacteria, mold and leaven). However, a harder treatment (temperatures over 80...Cwith times higher than 10 seconds) causes, after processing, a separation of the fatty substances of the juice (yellowish oil) that will be seen on the surface of the juice. This texture alteration reduces the acceptability of the product by consumer, because of its appearance.

As the losses in the organoleptic characteristics are much higher with more radical washing, without being able to reduce more the microbial load, the temperature of 80...Cand the time of 10 seconds were selected as the better washing conditions for the A ai fruit. However washing can t be considered efficient for the inactivating the peroxidase (enzymes more thermally resistant). Only at higher temperatures, at higher times, is a partial inactivity of this enzyme (up to 20%) acheived.

In concludion, the washing process is considered the primary step in the processing of the A ai juice, because it is able to reduce the microbial load before processing the A ai, without altering its texture significantly. Its maximum efficiency will be reached when that washing process is combined with the optimization of the other processing steps (cleaning, pasteurization). It will, then, be possible to conserve the A ai juice without altering its texture and taste significantly.

Thanking

We would like to thank the European Union —General Division XII for financial help (agreement with TS3-CT94-0300) and the Chemical Engineering Department of UFPa for providing us with material and labs.

NO References ?

- Cited in body, but not listed at end.
- See Açaí Summary of Information — it has references (the only ones obtained to date).

Note: Brazilian Studies are nearly impossible to obtain from USA & European libraries.

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Anthocyanins from fruits of açaí (*Euterpe oleracea*, Mart) and juçara (*Euterpe edulis*, Mart)

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Abstract *The anthocyanin pigments from fruits of açai (*Euterpe oleracea, *Mart) and juçara (*Euterpe edulis, *Mart) were extracted with 1% HCl in methanol, purified by descendent chromatography on Whatman 3MM paper and identified by chemical and physical analyses. The pigments in both fruits were identified as cyanidin 3-glucoside and cyanidin 3-rutinoside. The anthocyanin contents of the fruits of açai and juçara were 336 mg/100 g and 1347 mg/100 g, respectively.*

Keywords: açai, *Euterpe oleracea*, juçara, *Euterpe edulis*, anthocyanins, natural pigments.

Introduction

Açai (*Euterpe oleracea*, Mart) of the family Palmaceae, is a palm tree characteristic of the northern region of Brazil. It is characterized by the formation of sprouts and slightly curved plants with a high hard stipe and rings. The fruits are round or egg-like, forming bunches. The external colour is dark purple, almost black when mature, and the pulp is of the same colour. The seeds are hard (Bailey 1961; Cavalcante 1974; Braga 1976).

The mature fruits, when macerated in hot water and mixed with sugar give açaí wine, which is also purple. The wine is used as a food complement by the poor population of Amazonia, and is frequently their main meal, being mixed with tapioca or cassava flour (Cavalcante 1974; Braga 1976).

The juçara palm tree (*Euterpe edulis*, Mart) grows in the southern and south-western regions of Brazil and also belongs to the family Palmaceae. The fruits are very similar to those of açaí (Bailey 1961; Cavalcante 1974; Braga 1976).

The hearts-of-palm (palmito) are extracted from both species and represent a source of income for the regional economy, with a minor contribution to the export market.

As a consequence of the restrictions imposed on the use of certain synthetic dyes in foods, there is a need for red dyes in the food industry. The anthocyanins are the most important natural red pigments.

With the purpose of increasing the profit from regional fruits and as part of our screening programme for new sources of natural pigments from Brazilian plants, the

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anthocyanins of açai and juçara fruits were studied, mainly with the objective of providing new red dyes for foods.

Materials and methods

Samples

Fresh fruits from juçara (*Euterpe edulis*, Mart) and açai (*Euterpe oleracea*, Mart) were obtained from the Instituto Agronômico de Campinas (Estação Experimental de Ubatuba), Brazil. They were immediately frozen at −18°C and stored at this temperature until analysed. Maturity was determined by a visual inspection and based on their dark purple colour.

Extraction and purification of pigments

The pigments were extracted with 1% HCl in methanol and purified by descendent chromatography on Whatman 3 MM paper using 1% HCl and BAW twice as solvents (Harborne 1967; Francis *et al.* 1982; Draetta *et al.* 1985). The pigments were eluted separately in MAW.

The chromatographic solvents used are described in Table 1.

Spectral analysis

The spectral data (600–260 nm) were obtained with a HITACHI U-2000 spectrophotometer. The pigments were dissolved in methanol containing 0·1% HCl. The $AlCl_3$ shifts were determined by dissolving the purified pigment in 0·01% HCl in methanol and adding 2–3 drops of 5% $AlCl_3$ in ethanol.

Acid hydrolysis

Acid hydrolysis was performed by adding 2 ml 2N HCl to approximately 1 mg of pigment dissolved in MAW, and boiling for 30 min. After cooling, the aglycones

Table 1. Chromatographic solvents for anthocyanins

Abbreviation	Composition	Proportion (v/v)
BAW	*n*-Butanol : glacial acetic acid : water. Upper phase. Use within 1 h for Rf data.	4 : 1 : 5
BuHCl	*n*-Butanol : 2 N hydrochloric acid. Upper phase.	1 : 1
MAW	Methanol : glacial acetic acid : water	80 : 10 : 10
1% HCl	Concentrated hydrochloric acid : water	3 : 97
HAc-HCl	Water : glacial acetic acid : concentrated hydrochloric acid	82 : 15 : 3
Forestal	Glacial acetic acid : concentrated hydrochloric acid : water	30 : 3 : 10
Formic	Formic acid : concentrated hydrochloric acid : water	5 : 2 : 3
BPW	*n*-Butanol : pyridine : water	6 : 4 : 3
PEAW	*n*-Propanol : ethyl acetate : water	65 : 10 : 25
Sugar reagent	16·6 g *o*-phthalic acid and 91 ml aniline dissolved in 480 ml *n*-butanol, 480 ml diethyl ether and 40 ml water	

(anthocyanidins) were extracted using amyl alcohol, evaporated and spotted onto Whatman number 1 paper together with standard solutions of aglycones. The papers were run in forestal, formic and BAW solvents (see Table 1).

The remaining aqueous solution containing the sugar portions was dried and spotted onto Whatman number 1 paper for chromatography in BPW and PEAW (see Table 1). After development, the papers were dipped in the sugar reagent, dried and heated at 100°C for 5 min.

Controlled hydrolysis

Approximately 5 mg of pigment were dissolved in 2 ml MAW and 2 N HCl (2 ml) added. The resulting solution was heated to boiling in a water bath. The solution was sampled at intervals (0–32 min), each sample being spotted onto Whatman number 1 paper followed by development in BAW, BuHCl, 1% HCl and HAc-HCl (Table 1).

Anthocyanin content

The total anthocyanin content of the fruits was determined by the method described by Fuleki and Francis (1968), using extraction with ethanol-HCl.

The anthocyanin concentration was calculated as milligrams of cyanidin 3-glucoside per 100 g of fruit, assuming an extinction value ($E_{1\,cm}^{1\%}$) of 98·2.

Results and discussion

The most efficient purification mechanism appeared to be initial chromatography in 1% HCl, followed by successive chromatography in solvents based on butanol. The 1% HCl separation provided two pigments (designated 1 and 2), with pigment 2 present in slightly higher amounts. Each pigment was eluted with MAW, rechromatographed in BAW, eluted in MAW once again and rechromatographed in BAW.

From the spectral data presented in Table 2, no pigment showed fluorescence under UV light, indicating no substitution at the 5-position. These data were confirmed by the

Table 2. Spectral data for anthocyanins from açai (*Euterpe oleracea*, Mart) and juçara (*Euterpe edulis*, Mart) in 0·1% HCl in methanol

	Juçara		Açai	
	Pigment 1	Pigment 2	Pigment 1	Pigment 2
Max. visible wavelength (nm)	528	528	528	529
Max. wavelength UV (nm)	282	282	282	282
$A_{uv,max}/A_{vis,max}$	70·2	64·9	71·7	70·1
$A_{440}/A_{\lambda vis,max}$	28·1	28·3	25·7	24·9
UV fluorescence	No	No	No	No
Change in wavelength with addition of $AlCl_3$ (nm)	22·5	20·0	26·0	23·5

Table 3. Chromatographic data of pigments from açaí (*Euterpe oleracea*, Mart)

	Products of acid hydrolysis of anthocyanins					Controlled hydrolysis products					Anthocyanins			
	Aglycone (Rf × 100)			Sugar (RG, glucose = 100)			Solvents (RF × 100)				Solvents (RF × 100)			
	Forestal	Formic	BAW	BPW	PEAW	Compounds	HCl 1%	HAc-HCl	BAW	Bu HCl	HCl 1%	HAc-HCl	BAW	BuHCl
Pigment 1	47	14	71	100	101	1	9	49	37	17	10 (17)	37 (26)	40 (38)	20 (25)
						1a	3	30	71	73				
Pigment 2	48	15	70	100	101	2	19	65	35	19	23 (19)	52 (43)	37 (37)	19 (25)
				164	157	2a	8	53						
						2b	3	32	70	72				
Markers														
Poinsettia														
P1											10	36	38	20
P2											16	45	45	33
P3											22	49	36	17
P4											31	58	42	26
Cyanidin	48 (49)	16 (22)	71 (68)											
Pelurgonidin	62 (68)	25 (33)												
Galactose				82	99									
Arabinose				107	115									
Xylose				131	129									
Rhamnose				163	157									

Values in parentheses were reported by Harborne 1967; Francis *et al.* 1982; Draetta *et al.* 1985
Chromatographic solvents are described in Table 1

Table 4. Chromatographic data of pigments from juçara (*Euterpe edulis*, Mart)

	Products of acid hydrolysis of anthocyanins	Controlled hydrolysis products	Anthocyanins

Values in parentheses were reported by Harborne 1967; Franc[illegible]al. 1982; Draetta *et al.* 1985
Chromatographic solvents are described in Table 1

Table 4. Chromatographic data of pigments from juçara (*Euterpe edulis*, Mart)

	Products of acid hydrolysis of anthocyanins					Controlled hydrolysis products					Anthocyanins				
	Aglycone (Rf × 100)			Sugar (RG, glucose=100)			Solvents (RF × 100)				Solvents (RF × 100)				
	Forestal	Formic	BAW	BPW	PEAW	Compounds	HCl 1%	HAc-HCl	BAW	Bu HCl	HCl 1%	HAc-HCl	BAW	BuHCl
Pigment 1	37	15	72	100	100	1	6	44	35	22	11 (17)	41 (26)	35 (37)	25 (25)
						1a	1	15	72	73				
Pigment 2	37	15	71	100	100	2	15	60	37	18	27 (19)	53 (43)	37 (37)	24 (25)
				187	154	2a	6	43	—	—				
						2b	1	14	71	72				
Markers														
Poinsettia														
P1											11	43	40	24
P2											17	49	45	39
P3											22	53	37	22
P4											33	61	43	34
Cyanidin	37 (49)	15 (22)	71 (68)											
Pelargonidin	48 (68)	24 (33)												
Galactose				89	92									
Arabinose				127	115									
Xylose				156	131									
Rhamnose				187	153									

Values in parentheses were reported by Harborne 1967; Francis *et al.* 1982; Draetta *et al.* 1985
Chromatographic solvents are described in Table 1

ratio A440/Avis. max. The spectral curve of the two pigments showed only one peak in the UV region at 282 nm, characteristic of non-acylated anthocyanins. The addition of $AlCl_3$ showed a bathochromic effect in the visible spectra for the two pigments. This shift indicates the presence of free vicinal hydroxyl groups in the B ring.

Acid hydrolysis of the pigments yielded the same aglycone identified as cyanidin from the spectral data by chromatography comparing with standard solutions of cyanidin (Tables 3 and 4) and by comparison with data from the literature. The standard aglycones were prepared from eggplant (delphinidin) and Poinsettia bracts (cyanidin and pelargonidin) (Harborne 1967; Draetta *et al.* 1985).

Controlled acid hydrolysis of pigment 1 showed no intermediate pigment, indicating only one sugar which was identified as glucose (Tables 3 and 4). Controlled acid hydrolysis of pigment 2 indicated one intermediate substance (2a) similar to pigment 1 and identified as cyanidin 3-glucoside (Harborne 1967; Draetta *et al.* 1985). Acid hydrolysis of pigment 2 yielded two sugars, identified as glucose and rhamnose.

Comparing the results obtained in this study with those in the literature, pigments 1 and 2 from açai and juçara were identified as cyanidin 3-glucoside and cyanidin 3-rutinoside, respectively.

The amounts of anthocyanin in fruits of açaí and juçara were about 336 mg/100 g and 1347 mg/100 g, respectively, calculated as cyanidin 3-glucoside. These values are higher than those found in plums (*Prunus salicina*, L; 29·5 mg/100 g) and in anil trepador fruits (*Cissus sicyoides*, Linn; 119–4 mg/100 ml) (Toledo *et al.* 1983; Draetta *et al.* 1985).

The results allow consideration of the fruits of açaí and juçara as potential sources of natural pigments. However, more toxicological and stability studies are necessary to validate their approval as food dyes.

References

Bailey L. H. (1961) *The Standard Cyclopedia of Horticulture*. Vol. 1, p. 1176. New York: Macmillan.

Braga R. (1976) *Plantas do Nordeste, Especialmente do Ceará*, p. 8. Brasil: Escola Superior de Agricultura de Mossoró, Fortaleza.

Cavalcante P. B. (1974) *Frutas Comestíveis da Amazônia II*, p. 34–35. Belém, Brasil: Museu Paraense Emilio Goeldi, Publicações avulsas.

Draetta I. S., Iaderoza M., Baldini V. L. S. and Francis F. J. (1985) Antocianinas de ameixa (*Prunus salicina*, L.). *Ciência e Tecnologia de Alimentos* 5, 31–38.

Francis F. J., Draetta I. S., Baldini V. L. S. and Iaderoza M. (1982) New anthocyanins from *Tibouchina granulosa*. *Journal of the American Society for Horticultural Science* 107, 789–791.

Fuleki T. and Francis F. J. (1968) Quantitative methods for anthocyanins. 1. Extraction and determination of total anthocyanin in cranberries. *Journal of Food Science* 33, 72–77.

Harborne I. B. (1967) *Comparative Biochemistry of the Flavonoids*. London: Academic Press.

Toledo M. C. F., Reyes F. G. R., Iaderoza M., Francis F. J. and Draetta I. S. (1983) Anthocyanins from anil trepador (*Cissus sicyoides*, Linn). *Journal of Food Science* 48, 1368–1369.

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Accepted 8 May

MINISTERY of AGRICULTURE

Ministry's Cabinet

Legislation nº 136, March, 31ª. 1999.

IDENTITY AND QUALITY STANDARDS FOR AÇAÍ PULP

1 - Goal
Present regulation aims at the establishment of minimum identity and quality standards that should be fit by açaí integral pulp and açaí, to be used as beverage. This regulation does not apply to açaí pulp for any other use.

2 - Definition
Açaí integral pulp and açaí are products extracted from the eatable part of the fruit of the açaí tree (Euterpe oleracea, Mart.) after beeing softened by adequate technological method.

3 - Classification
Accordingly to the addition or not of water and its quantities, the product will be classified as:
3.1 - Açaí integral pulp is the pulp extracted from açaí without the addition of water, by mechanical methods, and without filtration. It may be submited to a physical conservation process.
3.2 - Thick or special Açaí (type A) is the pulp extracted with the addition of water, presenting more than 14 % of total solids and a very dense appearance.
3.3 - Medium or regular Açaí (type B) is the pulp extracted with the addition of water, presenting more than 11 % and up to 14 % of total solids and a dense appearance.
3.4 - Thin or popular Açaí (type C) is the pulp extracted with the addition of water, presenting more than 8 % and up to 11 % of total solids and a "not dense" appearance.

4 - Basic ingredients
The açaí integral pulp and the açaí are obtained from fresh, ripe and healthy fruits, accordingly to respective specifications, and without any dust, parasites or microorganisms that can make the product inappropriate to consumption.

5 - Optional ingredients:
5.1 - Water
The water used to the pulp extraction must be potable and follow its specific regulations.
5.2 - Acidulante
In the case of pasteurized açaí, mantained at room temperature it will be permited the addition of citric acid according to the "Good Manufacture Practices" regulations.

6 - Composition:
6.1 - The açaí integral pulp and the açaí must have its composition according to the fruit characteristics, with no alterations, mistures with other species fruits or any illegal practice.
6.2 – The açaí integral pulp must fit the following physical, chemical and organoleptic characteristics:
6.2.1 – Physical and chemical:

	minimum	maximum
Total solids (g/100 g.)	40.0	60.0
Proteins (g/100g)	5.0	-
Total lipids (g/100 gms)	20.0	-
Total carbohidrates (g/100 gms)	51.0	-

Obs: gms = grams of dried material (total solids)

6.2.2 – Organoleptical
Physical aspect: pasty, presenting dark points provenient from the skin that involves the fruit.
Colour: violet purple proper for the purple açaí pulp and light green for the green açaí pulp.
Smell: Characteristic

6.3 – The açaí (special, regular or popular) must fit the following physical, chemical and organoleptic characteristics:
6.3.1 - Physical and chemical:

	minimum	maximum
PH (g/100 g.)	4.00	6.20
Total acidity, in citric acid (g/100 g)	-	0.27 (popular) 0.35 (regular) 0.45 (special)
Total lipids	20.0	60.00
Proteins (g/100 gms)	8.0	-
Total sugars (g/100 gms)	-	40.0

Obs: gms = grams of dried material (total solids)

6.3.2 – Organoleptical
Physical aspect: The emulsion must stay stable even if heated up to 80°C.
Colour: violet purple proper for the purple açaí pulp and light green for the green açaí pulp.
Smell: Carachteristic

6.4 – The integral açaí pulp and the açaí may contain non edible parts of the fruit into the limits that doesn't change the quality and organoleptical carachteristics of the product.
6.4.1 - The integral açaí pulp and the açaí must fit all other physical, chemical, microscopical, microbiological and organoleptic characteristics fixed in the Identity and Quality Standards for general fruit pulp.

Mixing Instructions and Other Suggestions for Acai

Mixing Instructions:
Acai 14:1 dehydrate requires 13 parts water to 1 part dehydrate by weight.

A simple mix would be the 25 grams of Acai powder to 325ml cold water.

Blend at medium speed for at least 30 seconds to hydrate the powder adequately. If the mix can stand for a minute or so it will improve the texture.

Alternately: Mix 25 grams Acai powder with 200ml water and 125 ice, blend 30 seconds, let stand one minute.

Alternative Suggestions:
Using 125ml of milk or cream in stead of ice, makes a delicious smoothie.

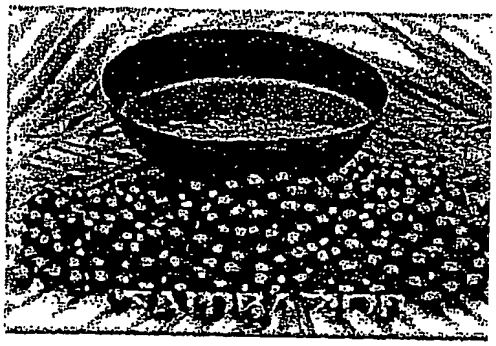
The taste of pure Acai is rather bland and the color is a very dark maroon. The addition of 1-2 tablespoons of sugar or other sweetener will awaken the flavor very nicely. The color can be made redder through addition of Vitamin C (an acid). The addition of red food color will also create a more appetizing or appealing appearance.

The addition of a banana to the mixture is a local favorite as well as a sprinkling of granola and garnishment of fruit.

Important: Do not mix Açaí in hot water. Açaí will oxidize quickly and should be mixed cold. Because Açaí is low in sugar and vitamin C there is very little to prevent oxidation / fermentation. The presence of both sugar and Vitamin C is recommended. Vitamin C will also make the mixture more red (and appealing).

Equipment:
Blender
Gram Scale
Milliliter measuring device

*A a (ah-sigh-ee) was recently "discovered" when ethno-botanical research found it to be the most nutritious fruit in the Amazon Rainforest. Since then, A ai has gone from an Amazonian secret to a dietary staple for Brazil's top athletic organizations.



Analysis reveals that A a contains:

- A remarkable concentration of antioxidants, to combat premature aging, with approximately 30 times the flavonoids of red wine.
- A rare synergy of cholesterol lowering monounsaturated fats, dietary fiber and phytosterols, providing radical heart protective and anti-carcinogenic potential.
- A complete amino acid complex in conjunction with valuable trace minerals, vital to proper muscle regeneration.

Utilizing a traditional Brazilian recipe, SAMBAZON A a is infused with pure Brazilian Guarana. (One serving of SAMBAZON A a contains less caffeine than a cup of coffee.)

Guarana is a revitalizing seed that has been used by Amazonian tribal warriors for centuries. The seed is ground into a powder and consumed before battle. Guarana is esteemed throughout the world for its ability to sustain strength and heighten awareness.

The result of this special blend is:

- Sustained energy and strength
- Heightened sense of awareness
- A massive attack on vitality-robbing free radicals
- The Rainforest is preserved simply and efficiently!


Guarana

A a is a dense source of anthocyanins (red-purple plant pigments with potent antioxidant properties)

- **Anthocyanins are a group of phytochemicals present in red wine, thought to contribute to the "French paradox" i.e. France has the lowest incidence of heart disease of any westernized society despite a prevalence of smoking and a diet high in saturated fat and cholesterol. A a contains 33 times the anthocyanins of red wine.**

- The primary anthocyanin in A a is cyanidin-3-glucoside. Cyanidin-3-glucoside has been found to be 3.5 times stronger than the predominant anthocyanin in red

wine (malvadin-3-glucoside) by an ORAC analysis (oxygen radical absorbance capacity).

The fatty acid ratio of A a closely resembles that of olive oil, which has long been thought to be a factor in the low incidence of heart disease in Mediterranean populations.

- A predominance of the monounsaturated lipid, oleic acid (omega-9), effectively lowers LDL (harmful cholesterol).
- The presence of the essential fatty acid, linoleic acid (omega-6), helps maintain healthy cell membranes.
- Fatty acids aid in the transport and absorption of fat-soluble vitamins i.e. vitamins A, E, D, and K.

A ai is rich in valuable sterols

- Sterols are components of plant cell membranes providing numerous benefits to the human body, namely the reduction of blood plasma cholesterol.
- Sterols affect the metabolism of prostaglanins, biologically active compounds found in the body, which reduce pain and inflammation.
- Sterols are currently being used to treat symptoms associated with BPH (benign prostate hyperplasia).
- Preliminary evidence suggests that beta-sitosterol (the predominant sterol in A ai) may prevent immune weakness resulting from severe physical stress.

A a is a significant source of fiber

- 5 grams of dietary fiber in one serving of A a .
- Fiber promotes a healthy digestive system. Low fiber in the American diet is thought to be a contributing factor to the high incidence of cancer and heart disease. Soluble fibers lower blood cholesterol; insoluble fibers are thought to help reduce the risk of developing certain types of cancers.

A a is mixed with Guarana

- Guarana increases metabolism, physical endurance and stamina.
- Guarana contains theobromine, the euphoriant in chocolate responsible for enhanced sense of well-being.
- Guarana is noted as being effective in the treatment of headache and colds. Other uses of Guarana include appetite suppression, pain relief, and as an aid to smoking cessation.
- Unlike many thermogenics, Guarana is not damaging to the heart. Clinical evidence has shown that Guarana actually protects the heart by inhibiting platelet aggregation (plaque formation), and by preventing the formation of blood clots.

*The information contained herein is derived from scientific research and is intended for educational purposes. These statements have not been reviewed by the Food and Drug Administration.

Acute oral toxicity study of 'Juçara fruit pulp - Freeze dried' with 14-day posttreatment observation period in the rat (limit test) (Study code: PCDL-0222)

SUMMARY

General information:

Single oral limit dose of 2,000 mg/kg body weight of 'Juçara fruit pulp - Freeze dried' (Lot number: 2208) was applied to rats orally by gavage. Animals were observed for lethality and toxic symptoms for 14 days.
Gross pathological examination was carried out on the 15th day.

Body weight:

The body weight of the animals corresponded to their species and age throughout the study.

Evaluation:

No death occurred after oral administration of 'Juçara fruit pulp - Freeze dried' at 2, 000 mg/kg dose.
No toxic clinical symptoms were observed.
Scheduled autopsy carried out on day 15 revealed no toxic gross pathological changes.

Conclusion

No adverse effects were noted at single oral dose of 2,000 mg/kg 'Juçara fruit pulp - Freeze dried' in male and female rats.

Contents

Staff in Charge

	Signature	Date
Director of the Laboratory:	István Financsek M.D., Ph.D.	16-12-2002
Head of the Toxicological Department, Study Director:	Susan Somfai-Relle Susan Somfai-Relle M.D., toxicologist	Dec 16, 2002
Deputy Study Director:	Dr. Tamara Varga Tamara Varga Ph.D., agronomist, toxicologist	Dec 16, 2002
Quality Assurance Unit:	Szilvia Karsai Szilvia Karsai Engineer of Quality Management	Dec 16, 2002.
Sponsor:	István Bara Managing Director COFOPEX Ltd.	Dec. 12, 2002
Monitoring Scientist:	Alexander G. Schauss Ph.D.	Dec 18, 2002

[redacted]

Acute oral toxicity study of 'Juçara fruit pulp - Freeze dried' with 14-day posttreatment observation period in the rat (limit test) (Study code: PCDL-0222)

Study Director's Statement

I hereby certify that this study report provides a true and complete record of the data generated and that the study was conducted in accordance with the Principles of Good Laboratory Practice as set forth in the following documents:

1. US Food and Drug Administration Title 21, Code of Federal Regulations, Part 58 Good Laboratory Practice Regulations for Nonclinical Laboratory Studies
2. Good Laboratory Practice Regulations (9/2001. EüM-FVM)
3. Hungarian Act 1998:XXVIII regulating animal protection.

Date Dec 16, 2002

Signature:

Dr Susan Somfai Relle

Susan Somfai-Relle, M.D.
Study Director

Acute oral toxicity study of 'Juçara fruit pulp - Freeze dried' with 14-day posttreatment observation period in the rat (limit test) (Study code: PCDL-0222)

Statement of the Quality Assurance Unit

This study has been inspected and the report audited by the Quality Assurance Unit of PCDL in compliance with the Principles of Good Laboratory Practice. As far as it can be reasonably established, the methods described and the results incorporated in the report accurately and completely reflect the raw data produced during this study.

Inspections concerning adherence to the protocol were performed:

Date Inspection / Audit	Type of inspection	Date of report to the Study Director	Management
24. 10. 2002	Protocol audit	25. 10. 2002	25. 10. 2002
12. 11. 2002	Treatment	12. 11. 2002	13. 11. 2002
11. 12. 2002	Draft report audit	11. 12. 2002	11. 12. 2002

Date: Dec 16, 2002 Signature: Szilvia Karsai

Mrs. Szilvia Karsai
Eng. Q. M.
Quality Assurance Unit

1. GENERAL INFORMATION

1.1. Title of the study

Acute oral toxicity study of 'Juçara fruit pulp - Freeze dried' with 14-day posttreatment observation period in the rat (limit test)

1.2. Objective of the study

To develop data on the potential toxicological effects of single oral administration of Juçara fruit pulp - Freeze dried in the rat. The test article is expected to be used as dietary supplement.

1.3. Type of the study

Preclinical toxicological study in compliance with the principles of the Good Laboratory Practice Regulations for Nonclinical Laboratory Studies of the United States Food and Drug Administration and the Hungarian Act 1998: XXVIII. regulating animal protection. Limit test.

1.4. Deviations from the Study Protocol

1.4.1. **2.3. Characteristics of substance T 61 used for extermination** Manufacturer:
Original protocol: Hoechst Veterinär GmbH
Final Report: Intervet International
Reason: The name of the manufacturer has been changed.

1.4.2. **6.1. Lethality**
Original protocol: Observations are made for 4 hours following treatment and twice daily thereafter.
Final Report: Observations were made for 4 hours following treatment and twice daily thereafter at the beginning and at the end of the working day as well as once at weekends, until the morning of the 15th day. The time of death should have been recorded as accurately as possible.
Reason: Procedures have been described more precisely than originally

1.4.3. **6.2. General state, external appearance, behavior, and clinical symptoms**
Original protocol: ..... during the subsequent period, animals are checked twice daily for physical signs of toxicity.
Final Report: .....During the subsequent period, they were checked daily twice until the morning of the 15th day except for week-ends, when they were checked once.
Reason: Procedures have been described more precisely than originally

2. TEST AND REFERENCE ARTICLES

2.1. Characteristics of the test article

Name of the article:	Juçara fruit pulp - Freeze dried
Botanical name:	Euterpe Edulis, Mart, Family: Palmae
Plant part used:	Fresh Frozen Fruit Pulp
Manufacturer:	Greater Continents do Brasil Ltda. Rua Alabastro, 55-112, Aclimação 01531-010 São Paulo, SP Brasil

Lot # :	2208
Identification number in PCDL:	2002/22886
Residual moisture:	max. < 2%
Physical characteristics:	dark purple granular freeze dried powder with characteristic odor and flavor, hygroscopic
Storage conditions:	refrigerated according to c. USP (2-8°C, humidity not controlled), re-sealed quickly if opened
Harvest date:	August, 2002
Production date:	October, 2002
Expiry:	October 01, 2003

2.1.1. Microbiological analysis

Microbiological limit test according to c. USP was carried out by the Microbiological Department of PCDL.

2.2. Characteristics of the article used for suspending the test article

2.2.1. Name:methylcellulose

Manufacturer:	Sigma
Batch number:	127H1066
Storage conditions:	at room temperature
Expiry:	02. 2003

2.2.2. Name: distilled water

Manufactured by:	PCDL
Batch number:	A0010102
Storage conditions:	at room temperature
Expiry:	03. 2003

2.3. Characteristics of article used for overanesthesia before necropsy

Name:	T 61.
Ingredients:	0.2 g embutramide, 0.005 g tetracaine hydrochloride, and 0.05 g mebezonium ioide per ml
Manufacturer:	Intervet International
Batch number:	09W008
Storage conditions:	at room temperature, in safety box for poisonous drugs
Expiry date:	05. 2006
Dose:	0.1 ml / 100 g body weight

2.4. Formulation of the test article

The necessary amount of the test article was weighed and suspended in 1% methylcellulose containing solution not earlier than 30 min before administration.

The following suspension was prepared:

Nominal dose 2000 mg/kg: 5.0 g Juçara fruit pulp ad 50 ml of 1% methyl-cellulose solution

Suspension was stirred during treatment with a Radelkis magnetic stirrer type OP-951.

2.4.1. Concentration control of the formulated test article

Samples of the formulated test substance were taken for check of the concentration and homogeneity. Concentration and homogeneity check was performed by gravimetry.

The concentration of all three samples measured in triplicates of the upper, intermediate, and lower parts of the suspension (homogeneity check) were within the acceptable ± 10% limits i.e. upper: +9.4 ± 4.2%, intermediate: +9.4 ± 4.6%, lower: +6.6 ± 2.0%.

2.4.2. Stability control of the test article

Stability control of the test article is the Sponsor's responsibility

3. TEST SYSTEM

3.1. Animals

Species / Strain:	Sprague Dawley rat, Crl:CD BR
Age at arrival:	6-7 weeks
Body weight at arrival:	
	males: 143.8 - 151.9 g
	females: 144.2 - 161.6 g

A pool of animals ordered: 30 (15 males, 15 females)

Number of animals involved in the study: 20 (10 males, 10 females)

3.1.1. Supplier

Charles River Hungary Ltd.

H-1078 Budapest, István u. 11.

3.1.2. Hygienic class

SPF at arrival, kept in conventional environment during the study.

3.2. Reason for the selection of species

The rat is commonly used for toxicological studies in accordance with international recommendations. The Sprague Dawley strain is a well-known laboratory model with sufficient historical data.

3.3. Identification and housing of animals

The animals were identified by ear numbering technique and housed in cages by five of the same sex. The cages were labelled with tags indicating the I.D. numbers of the rats, the study code, the group identification, route of administration, sex and the starting and ending dates of the experimental period.

3.4. Housing conditions

Hygienic level:	conventional
Type of animal cages:	type II macrolone
Size of cage: H x W x D :	17.5 cm x 22.5 cm x 37.5 cm
Cleaning:	by changing the bottom of the cages three times a week

Number of animals per cage: 5

Number of animal keeping room: 123

3.4.1. Environmental conditions

Air exchange:	approximately 15 times/hour
Temperature:	22 ± 3°C
Relative humidity:	30 - 70 %
Lighting:	artificial, 12 hour light-dark cycles.

The temperature and the relative humidity were continuously recorded.

3.4.2. Feed

Free access to standardized rat and mouse diet VRF-1 except for the overnight fasting period prior to treatment, during the treatment and for the two first hours of the postreatment observation.

The composition of the diet is controlled by the Manufacturer Altromin GmbH, D-4937 Lage/Lippe Lange Str. 42.

The diet was identified by the date of manufacturing (30. 09. 2002), stability: 4 months.

3.4.3. Drinking

Rats had free access to tap water via drinking bottles. Drinking water is checked monthly by the Microbiological Department of PCDL.

3.5. Acclimatization period

The animals were observed for 5 days prior to the treatment. Only healthy animals, free from any clinical symptom were used in the study.

3.6. Randomization

Grouping of the animals was made with a random table generated by a computer. The animals were randomly assigned to groups on the basis of their body weight, so that the distribution of the body weights in the individual groups were similar.

4. EXPERIMENTAL DESIGN

4.1. Dose levels, group division

Group number	Treatment	Dose	Number of animals		Identification numbers	
		mg/kg	Males	Females	Males	Females
1	Juçara fruit pulp	2,000	10	-	871-880	-
2	Juçara fruit pulp	2,000	-	10	-	881-890

4.2. Reason for dose selection

The expected human daily dose of Juçara fruit pulp is approx. 1000 mg per day which corresponds to 14 mg/kg body weight of an adult (70 kg) or 50 mg/kg for a 4 years old child (20 kg). The 2000 mg/kg limit dose applied in this study corresponds to 140 times of the daily dose if consumed by an adult or 40 times of it if 5 g is calculated for a child's body weight.

5. ADMINISTRATION

5.1. Route of administration and reason for the selection

Application was oral by gavage. The route of application was selected in compliance with international guidelines. The oral route is the anticipated route of human exposure to the test article.

5.2. Frequency and duration of application

Single dose.

5.3. Volume of application

The test article was administered in a volume of 20 ml/kg body weight.

5.4. Duration of the experimental period

5 days of acclimatization, treatment's day, 14 days posttreatment observation period including the treatment's day, and the 15th day: necropsy.

6. OBSERVATIONS, EXAMINATIONS

6.1. Lethality

Observations were made for 4 hours following treatment and twice daily thereafter at the beginning and at the end of working days as well as once at weekends until the morning of the 15th day. The time of death should have been recorded as accurately as possible.

6.2. General state, external appearance, behaviour, and clinical symptoms

Careful clinical observation of the rats was carried out once before the exposure, then, after the treatment for 6 hours continuously. During the subsequent period, they were checked daily twice until the morning of the 15th day except for week-ends, when they were checked once. Signs to be observed included changes in skin, fur, eyes and visible mucous membranes; occurrence of secretions and excretions and autonomic activity (e.g. lacrimation, piloerection, diarrhea, pupil size, unusual respiratory pattern). Furthermore, potential changes in gait, posture and response to handling as well as the presence of somnolence, trembling, clonic or tonic movements, stereotypes or bizarre behaviour were recorded.

6.3. Body weight

Animals were weighed at arrival in the laboratory, on the day of randomization, on the day of treatment, as well as on the 2nd, 8th, and 15th day of the experiment prior to the necropsy.

7. NECROPSY AND HISTOLOGICAL EXAMINATION

7.1. Necropsy

All surviving rats on completion of the posttreatment observation period were exterminated under T61 overanaesthesia and autopsied. External and internal status were carefully observed and recorded.

No microscopic examination of organs was performed.

[redacted]

8. EVALUATION, STATISTICAL ANALYSIS

Groups of males and females were evaluated separately.

8.1. Parametric values

Individual body weight changes were calculated and tabulated. Mean values and standard deviations were calculated of the body weights and body weight changes.

8.2. Non parametric values (lethality and clinical symptoms)

The incidence of lethality, clinical symptoms, and gross findings were tabulated.

9. PROCEDURES

The experiments were performed according to the current Standard Operating Procedures of the Department of Toxicology of the Pharmaceutical Control and Development Laboratory Co. Ltd.

10. ANIMAL PROTECTION

In the interests of animal welfare the unnecessary use of animals was avoided. To order the mild extermination of unambiguously moribund animals was the responsibility of the study director. The present method (limit test) uses a reduced number of experimental animals in comparison to other known and acknowledged acute toxicity tests.

11. DATA RECORDING AND ARCHIVATION

All original data are maintained, as dictated by the Standard Operating Procedures, on appropriate forms as follows:

- Test Compound weighing
- Animal room logbook
- Body weight logbooks
- Lethality and Clinical observations logbooks
- Postmortem records

The data obtained in the course of the study were collected in a Study File. The Study Protocol, all data generated during and as a result of the study, the documents and all information in connection with the study, a control sample of the test article and the Final Report will be stored at least for 15 years in the Archives of the PCDL then offered to the Sponsor.

12. SCHEDULE OF THE STUDY

Arrival of the animals:	November 7, 2002
Randomization:	November 11, 2002
Treatment day:	November 12, 2002
Necropsy:	November 26, 2002

13. RESULTS

13.1. Lethality

(see Table 1. and Appendices 1.1.-1.2.)

No death occurred following the single oral administration of 2, 000 mg/kg dose of 'Juçara fruit pulp - Freeze dried' to rats. All males and females survived until the end of the 14-day observation period.

13.2. Clinical symptoms

(see Table 2. and Appendices 2.1.-2.2.)

No toxic symptoms were observed on the day of application and during the 14-day posttreatment period at any group of the treated animals.

13.3. Body weights

(see Tables 3.1.-3.2. and Appendices 3.1.-3.4.)

The body weight and the body weight gain of the animals corresponded to their species and age throughout the study.

13.4. Gross pathology

(see Table 4. and Appendices 4.1.-4.2.)

All animals survived until the scheduled autopsy on day 15 and all proved to be free of toxic pathological changes.

[redacted]

14. EVALUATION

No death occurred after single oral application of 2, 000 mg/kg 'Juçara fruit pulp - Freeze dried' dose.

No toxic clinical symptoms occurred.

Scheduled autopsy at day 15 revealed no toxic gross pathological changes.

Conclusion

No adverse effects were noted at single oral dose of 2,000 mg/kg 'Juçara fruit pulp - Freeze dried' in male and female rats.

Dr Susan Gompfa-Nelle

Study Director Dec 16, 2002

[redacted]

Tables

[redacted]

Acute oral toxicity study of 'Juçara fruit pulp - Freeze dried' with 14-day posttreatment observation period in the rat (limit test)

Table 1.

Lethality

Post-treatment observation period (14 days)

	Group 1	Group 2
	MALES	FEMALES
Treatment	death / number of animals	
Juçara fruit pulp; 2,000 mg/kg, po.	0/10	0/10

Table 2.

Clinical Symptoms

Post-treatment observation period (14 days)

	Group 1	Group 2
	MALES	FEMALES
Treatment	symptom / number of animals	
Juçara fruit pulp; 2,000 mg/kg, po.	0/10	0/10

Acute oral toxicity study of 'Juçara fruit pulp - Freeze dried' with 14-day posttreatment observation period in the rat (limit test)

Table 3.

Body Weights

MALES

Group 1 Treatment	Body weights [g]					
	Day of arrival	Day of randomi-zation	Day 1 prior to treatment	Day 2	Day 8	Day 15
Juçara fruit pulp; 2,000 mg/kg, po.						
Group size:	10	10	10	10	10	10
Mean:	148.1	198.6	181.8	195.6	255.5	313.8
± S.D.:	3.37	6.33	6.84	14.97	8.98	19.09

FEMALES

Group 2 Treatment	Body weights [g]					
	Day of arrival	Day of randomi-zation	Day 1 prior to treatment	Day 2	Day 8	Day 15
Juçara fruit pulp; 2,000 mg/kg, po.						
Group size:	10	10	10	10	10	10
Mean:	152.6	177.8	162.7	179.6	204.9	227.2
± S.D.:	6.25	7.46	7.13	9.61	5.81	19.25

[redacted]

Acute oral toxicity study of 'Juçara fruit pulp - Freeze dried'
with 14-day posttreatment observation period in the rat (limit test)

Table 3.2.

Body Weight Changes

MALES

Groups	Body weight changes [g]		
	Day 1	Day 2 through Day 7	Day 8 through Day 14
Juçara fruit pulp; 2,000 mg/kg, po.			
Group size:	10	10	10
Mean:	13.8	60.0	58.3
± S.D.:	10.76	10.28	20.36

FEMALES

Groups	Body weight changes [g]		
	Day 1	Day 2 through Day 7	Day 8 through Day 14
Juçara fruit pulp; 2,000 mg/kg, po.			
Group size:	10	10	10
Mean:	16.9	25.4	22.3
± S.D.:	4.92	5.62	14.59

Acute oral toxicity study of 'Juçara fruit pulp - Freeze dried' with 14-day posttreatment observation period in the rat (limit test)

Table 4.

Gross Pathology Findings

	Group 1		Group 2	
	MALES		FEMALES	
Treatment	external	internal	external	internal
	finding / number of animals			
Juçara fruit pulp; 2,000 mg/kg, po.	0/10	0/10	0/10	0/10

**Acute oral toxicity study of 'Juçara fruit pulp - Freeze dried'
with 14-day posttreatment observation period in the rat (limit test)**

Appendices

[redacted]

Acute oral toxicity study of 'Juçara fruit pulp - Freeze dried' with 14-day posttreatment observation period in the rat (limit test)

Appendix 1.1.

Individual Data of Lethality

MALES

Group / Animal code	DAYS OF OBSERVATION PERIOD														
	Day 1*	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15
Group 1: Juçara fruit pulp; 2,000 mg/kg, po.															
871	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
872	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
873	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
874	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
875	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
876	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
877	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
878	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
879	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
880	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Remark: 0 = No Lethality

* Day 1 = Treatment's day

Acute oral toxicity study of 'Juçara fruit pulp - Freeze dried'
with 14-day posttreatment observation period in the rat (limit test)

Appendix 1.2.

Individual Data of Lethality

FEMALES

Group / Animal code	DAYS OF OBSERVATION PERIOD														
	Day 1*	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15
Group 2: Juçara fruit pulp; 2,000 mg/kg, po.															
881	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
882	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
883	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
884	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
885	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
886	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
887	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
888	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
889	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
890	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Remark: 0 = No Lethality

* Day 1 = Treatment's day

[redacted]

Acute oral toxicity study of 'Juçara fruit pulp - Freeze dried' with 14-day posttreatment observation period in the rat (limit test)

Appendix 2.1.

Individual Clinical Symptoms

MALES

Group / Animal code	DAYS OF OBSERVATION PERIOD														
	Day 1*	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15
Group 1: Juçara fruit pulp; 2,000 mg/kg, po.															
871	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF
872	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF
873	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF
874	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF
875	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF
876	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF
877	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF
878	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF
879	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF
880	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF

Remark: SF = Symptom Free

* Day 1 = Treatment's day

[redacted]

Acute oral toxicity study of 'Juçara fruit pulp - Freeze dried' with 14-day posttreatment observation period in the rat (limit test)

Appendix 2.2.

Individual Clinical Symptoms

FEMALES

Group / Animal code	DAYS OF OBSERVATION PERIOD														
	Day 1*	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15
Group 2: Juçara fruit pulp; 2,000 mg/kg, po.															
881	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF
882	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF
883	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF
884	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF
885	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF
886	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF
887	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF
888	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF
889	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF
890	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF	SF

Remark: SF = Symptom Free

* Day 1 = Treatment's day

Acute oral toxicity study of 'Juçara fruit pulp - Freeze dried' with 14-day posttreatment observation period in the rat (limit test)

Appendix 3.1.

Individual Body Weights

MALES

Group / Animal code	Body weights [g]					
	Day of arrival	Day of randomization	Day 1 prior to treatment	Day 2	Day 8	Day 15
Group 1: Juçara fruit pulp; 2,000 mg/kg, po.						
871	146.3	208.2	191.5	213.4	265.7	328.7
872	151.9	205.3	185.1	208.7	255.1	314.2
873	147.9	202.5	188.8	209.3	265.9	310.5
874	143.8	202.2	178.5	204.7	263.5	323.5
875	148.9	200.1	180.3	207.1	261.5	332.7
876	145.6	198.9	178.3	181.4	258.8	284.3
877	153.9	196.2	188.3	194.0	248.8	322.0
878	146.9	192.6	183.3	186.7	242.9	334.9
879	144.3	190.7	171.3	174.0	250.8	279.6
880	151.2	189.2	172.5	176.2	242.2	307.3
Group size:	10	10	10	10	10	10
Mean:	148.1	198.6	181.8	195.6	255.5	313.8
± S.D.:	3.37	6.33	6.84	14.97	8.98	19.09

Acute oral toxicity study of 'Juçara fruit pulp - Freeze dried' with 14-day posttreatment observation period in the rat (limit test)

Appendix 3.2.

Individual Body Weights

FEMALES

Group / Animal code	Body weights [g]					
	Day of arrival	Day of randomi-zation	Day 1 prior to treatment	Day 2	Day 8	Day 15
Group 2: Juçara fruit pulp; 2,000 mg/kg, po.						
881	154.2	193.4	174.4	197.1	216.0	263.8
882	159.9	185.6	175.0	187.4	211.4	256.3
883	144.2	182.0	167.0	184.4	204.0	237.1
884	161.6	178.1	162.9	185.4	206.5	223.1
885	158.6	178.1	160.0	185.1	207.2	225.5
886	150.3	175.9	159.4	173.4	197.0	208.9
887	155.7	172.3	156.3	167.6	200.4	216.2
888	148.8	171.4	158.6	174.1	200.2	211.5
889	147.5	170.7	157.5	169.7	200.2	218.1
890	145.3	170.5	155.8	171.5	206.5	211.5
Group size:	10	10	10	10	10	10
Mean:	152.6	177.8	162.7	179.6	204.9	227.2
± S.D.:	6.25	7.46	7.13	9.61	5.81	19.25

Acute oral toxicity study of 'Juçara fruit pulp - Freeze dried'
with 14-day posttreatment observation period in the rat (limit test)

Appendix 3.3.

Individual Body Weight Changes *

MALES

Groups / Animal code	Body weight changes [g]		
	Day 1	Day 2 through Day 7	Day 8 through Day 14
Group 1: Juçara fruit pulp; 2,000 mg/kg, po.			
871	21.9	52.3	63.0
872	23.6	46.4	59.1
873	20.5	56.6	44.6
874	26.2	58.8	60.0
875	26.8	54.4	71.2
876	3.1	77.4	25.5
877	5.7	54.8	73.2
878	3.4	56.2	92.0
879	2.7	76.8	28.8
880	3.7	66.0	65.1
Group size:	10	10	10
Mean:	13.8	60.0	58.3
± S.D.:	10.76	10.28	20.36

* Differences calculated from body weights weighed on Days 1 and 2, Days 2 and 8 as well as Days 8 and 15, respectively.

Acute oral toxicity study of 'Juçara fruit pulp - Freeze dried'
with 14-day posttreatment observation period in the rat (limit test)

Appendix 3.4.

Individual Body Weight Changes *

FEMALES

Groups / Animal code	Body weight changes [g]		
	Day 1	Day 2 through Day 7	Day 8 through Day 14
Group 2: Juçara fruit pulp; 2,000 mg/kg, po.			
881	22.7	18.9	47.8
882	12.4	24.0	44.9
883	17.4	19.6	33.1
884	22.5	21.1	16.6
885	25.1	22.1	18.3
886	14.0	23.6	11.9
887	11.3	32.8	15.8
888	15.5	26.1	11.3
889	12.2	30.5	17.9
890	15.7	35.0	5.0
Group size:	10	10	10
Mean:	16.9	25.4	22.3
± S.D.:	4.92	5.62	14.59

* Differences calculated from body weights weighed on Days 1 and 2, Days 2 and 8 as well as Days 8 and 15, respectively.

Acute oral toxicity study of 'Juçara fruit pulp - Freeze dried'
with 14-day posttreatment observation period in the rat (limit test)

Appendix 4.1.

Gross Pathology Findings

MALES

Group / Animal code	Day 15	
	external	internal
Group 1: Juçara fruit pulp; 2,000 mg/kg, po.		
871	No Finding	No Finding
872	No Finding	No Finding
873	No Finding	No Finding
874	No Finding	No Finding
875	No Finding	No Finding
876	No Finding	No Finding
877	No Finding	No Finding
878	No Finding	No Finding
879	No Finding	No Finding
880	No Finding	No Finding

„No Finding" stays here for the following text:

external: Animal of average development. Skin, fur, visible mucous membranes are intact.

internal: organs are without pathological changes

[redacted]

Acute oral toxicity study of 'Juçara fruit pulp - Freeze dried' with 14-day posttreatment observation period in the rat (limit test)

Appendix 4.2.

Gross Pathology Findings

FEMALES

Group / Animal code	Day 15	
	external	internal
Group 2: Juçara fruit pulp; 2,000 mg/kg, po.		
881	No Finding	No Finding
882	No Finding	No Finding
883	No Finding	No Finding
884	No Finding	No Finding
885	No Finding	No Finding
886	No Finding	No Finding
867	No Finding	No Finding
888	No Finding	No Finding
889	No Finding	No Finding
890	No Finding	No Finding

„No Finding" stays here for the following text:

external: Animal of average development. Skin, fur, visible mucous membranes are intact.

internal: organs are without pathological changes

Greater Continents do Brasil Ltda.
Rua Alabastro, 55 - 112
Aclimação
01531-010 São Paulo, SP Brasil
CNPJ # 02.935.969/0001-80

Product Specification Sheet

Product:	Juçara fruit pulp – Freeze dried				
Code #:	GCB –J2001.L	*lot #:*	2208	*HTC:*	1302.19.00

Common Name:	*Juçara*
Botanical Name:	*Euterpe edulis, Mart*
Botanical Family:	Palmae
Plant part used:	Fresh Frozen Fruit Pulp

Analysis	*Specification*	*Results*
Appearance:	Pulp powder	Conforms
Color	Dark purple	Conforms
Odor:	Characteristic	Conforms
Flavor:	Characteristic	Conforms
Residual moisture	Max <2%	Conforms
Excipient:	None	Conforms

Microbiological Analysis			
Analysis	Specification	Result	
Bacteria aerobica Total	Max 10^5/g	NA	
Coliformes 35° C/g		NA	
Coliformes 45° C/g		NA	
E. Coli	absent/g	NA	
Mold & Yeast		NA	
Conclusion			
Product is appropriate for consumption			

Harvest Date: August 2002
Production Date: October 01,2002

Thomas A. Gara
President/CEO
Greater Continents do Brasil Ltda.

ORSZÁGOS GYÓGYSZERÉSZETI INTÉZET
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HUNGARY

Budapest, 23rd May 2002
No.: 5165/48/2002
Our ref.: Dr Éva Iván/KK
Annex:
Subject:

STATEMENT OF GLP COMPLIANCE

Date of inspection: 16-17 April, 2002

Assessment of conformity with GLP Principles has been performed according to Council Directives 87/18/EEC and 88/320/EEC and Commission Directives 1999/11/EC and 1999/12/EC.

According to the criteria specified in paragraph 5 of Article 3 of the Joint Decree No. 9/2001. (III.30.) EüM-FVM of the Minister of Health and the Minister of Agriculture and Regional Development on the application and verification of good laboratory practice the Director-General of the National Institute of Pharmacy certifies that the test facility

Pharmaceutical Control and Development Laboratory Co. Ltd. (PC & DL)
H-1149 Budapest, Mexikói út 9., Hungary

is able to carry out ***toxicity studies and safety pharmacology testing*** in compliance with the Principles of Good Laboratory Practice as established by the OECD and the European Community.

ORSZÁGOS GYÓGYSZERÉSZETI INTÉZET

Prof Sándor Kerpel-Fronius MD., DSc.
Director-General

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Development and Validation of Oxygen Radical Absorbance Capacity Assay for Lipophilic Antioxidants Using Randomly Methylated β-Cyclodextrin as the Solubility Enhancer

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We recently reported the improved oxygen radical absorbance capacity (ORAC) assay using fluorescein (FL) as the fluorescent probe. The current $ORAC_{FL}$ assay is limited in hydrophilic antioxidant due to the aqueous environment of the assay. Lipophilic antioxidants mainly include the vitamin E family and carotenoids, which play a critical role in biological defense systems. In this paper, we expanded the current $ORAC_{FL}$ assay to lipophilic antioxidants. Randomly methylated β-cyclodextrin (RMCD) was introduced as the water solubility enhancer for lipophilic antioxidants. Seven percent RMCD (w/v) in a 50% acetone−H_2O mixture was found to sufficiently solubilize vitamin E compounds and other lipophilic phenolic antioxidants in 75 mM phosphate buffer (pH 7.4). This newly developed ORAC assay (abbbreviated $ORAC_{FL-LIPO}$) was validated through linearity, precision, accuracy, and ruggedness. The validation results demonstrate that the $ORAC_{FL-LIPO}$ assay is reliable and robust. For the first time, by using 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid as a standard (1.0), the ORAC values of α-tocopherol, (+)-γ-tocopherol, (+)-δ-tocopherol, α-tocopherol acetate, tocotrienols, 2,6-di-*tert*-butyl-4-methylphenol, and γ-oryzanol were determined to be 0.5 ± 0.02, 0.74 ± 0.03, 1.36 ± 0.14, 0.00, 0.91 ± 0.04, 0.16 ± 0.01, and 3.00 ± 0.26, respectively. The structural information of oxidized α-tocopherol obtained by liquid chromatography/mass spectrometry reveals that the mechanism for the reaction between the vitamin E and the peroxyl radical follows the hydrogen atom transfer mechanism, which is in agreement with the notion that vitamin E is the chain-breaking antioxidant.

KEYWORDS: ORAC; cyclodextrin; LC/MS; lipophilic antioxidants; vitamin E; chain breaking; hydrogen atom transfer

INTRODUCTION

Antioxidants can be physically classified by their solubility into two groups (*1*) (i) hydrophilic antioxidants, such as vitamin C and the majority of polyphenolic compounds, and (ii) lipophilic antioxidants, mainly including vitamin E and carotenoids. Similar to hydrophilic antioxidants, lipophilic antioxidants play an important role in a wide spectrum of biochemical and physiological processes. Of primary interest is their optimal antioxidant activity in vitro and in vivo. Unlike hydrophilic antioxidants, which do not accumulate in the body and are excreted in the urine, lipophilic antioxidants penetrate the lipoprotein cell membrane more easily and therefore reach a higher level of bioavailability (*2*). Although lipophilic antioxidants are highly bioavailable, it is not a trivial task to accurately measure their antioxidant activity in vitro. There are a number of methods available for measuring antioxidant activity such as the oxygen radical absorbance capacity (ORAC) fluorescein (*FL*) assay from our laboratory; all of these methods are conducted in an aqueous system, as such, none of which are suitable for lipophilic antioxidants. Without knowing the actual effectiveness of the lipophilic antioxidants, consumers can be exposed to unsafe concentrations or ineffective dosages. We overcame this obstacle by introducing randomly methylated β-cyclodextrin (RMCD) as a molecular host to enhance the solubility of lipophilic antioxidants in aqueous solution. Specifically, cyclodextrins (CDs) are cyclic (α-1,4)-linked oligosaccharides of α-D-gluco-pyranose containing a relatively hydrophobic (fatlike) central cavity and hydrophilic (waterlike) outer surface. This property of CD has made it increasingly popular as a vehicle for enhancing the solubility of fat soluble compounds in an aqueous environment in pharmaceutical and food industries (*3*, *4*). In this paper, we will report the development and validation of a new ORAC assay specific for lipophilic antioxidant activity. To our knowledge, it is the first time that vitamin E and other common lipophilic phenolic antioxidants were measured using the ORAC assay.

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MATERIALS AND METHOD

Chemicals and Apparatus. RMCD was purchased from Cyclolab R&D Ltd. (Budapest, Hungary). FL and 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox) were purchased from Aldrich (Milwaukee, WI). 2,2′-Azobis (2-amidino-propane) dihydrochloride (AAPH) was obtained from Wako Chemicals USA (Richmond, VA). γ-Oryzanol was purchased from TCI America (Portland, OR). Nutriene (tocotrienols) was obtained from Eastman Chemicals Company (Kingsport, TN). 4-Difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a- diaza-*s*-indacene-3-undecanoic acid (BODIPY 581/591 C11) was purchased from Molecular Probes, Inc. (Eugene, OR). All other standards were commercially available form Sigma or Aldrich. All ORAC analyses were performed on a COBAS FARA II analyzer (Roche Diagnostic System Inc., Branchburg, NJ) using an excitation wavelength of 493 nm and an emission filter of 515 nm. The photobleaching experiment of BODIPY 581/591 C11 was carried out in a Bio-Tek fluorescence microplate reader FL600 (Bio-Tek, Winooski, VT). The identification of oxidized Trolox and α-tocopherol induced by AAPH was performed by a HP 1100 high-performance liquid chromatography (HPLC) system (Hewlett-Parkard, Palo Alto, CA) coupled with a Finigan LCQ ion trap mass spectroscopic detector (ThermoFinigan, San Jose, CA).

Sample Preparation. Approximately 0.5 g of sample was dissolved in 20 mL of acetone. An aliquot of sample solution was appropriately diluted with 7% RMCD solvent (w/v) made in a 50% acetone−water mixture (v/v) and was shaken for 1 h at room temperature on an orbital shaker at 400 rpm. The sample solution was ready for analysis after further dilution with 7% RMCD solvent.

Automatic ORAC Assay. The automated ORAC assay was carried out on a COBAS FARA II spectrofluorometric centrifugal analyzer. The procedure was based on a previous report of Ou and co-workers (*5*). With the exception of samples and Trolox standards, which were made in 7% RMCD solvent, all other reagents were prepared at 75 mM phosphate buffer (pH 7.4). In the final assay mixture (0.4 mL total volume), FL (6.3×10^{-8} M) was used as a target of free radical attack and AAPH (1.28×10^{-2} M) was used as a peroxyl radical generator. Seven percent RMCD was used as the blank, and Trolox (12.5, 25, 50, and 100 μM) was used as the control standard. The analyzer was programmed to record the fluorescence of FL every minute after the addition of AAPH. All measurements were expressed relative to the initial reading. Final results were calculated using the differences of areas under the FL decay curves between the blank and a sample. These results were expressed as micromoles Trolox equivalent (TE).

Competitive Reaction of Trolox and α-Tocopherol with AAPH. A total of 0.5 mL of 0.5 mM Trolox and 0.5 mL of 0.5 mM α-tocopherol (prepared in 7% RMCD) were mixed in a 1.5 mL HPLC sample vial followed by the addition of 20 μL of 640 mM AAPH; the reaction solution was incubated at 37 °C in an autosampler. At every 10 min interval, 5 μL of reaction solution was injected into a Zorbax C18 column (Hewlett-Packard, Palo Alto, CA) (2.1 mm × 150 mm, 3 μm) until the reaction was completed. The mobile phase was 70% methanol with a flow rate of 0.3 mL/min, and the UV detector was set at 280 nm.

Characterization of Oxidized Products of Trolox Induced by AAPH. A mixture of 0.2 mL of 2.0 mM Trolox and 0.8 mL of 640 mM AAPH was incubated at 37 °C for 30 min. The reaction products were separated by a Zorbax C18 column (2.1 mm × 150 mm, 3 μm) at 37 °C with a mobile phase of 70% methanol at a flow rate of 0.3 mL/min, and the UV detector was set at 280 nm. The oxidized products were characterized using a Finnigan LCQ ion trap mass spectrometer equipped with an API chamber and an electrospray ionization (ESI) source. The ionization was negative ion mode, and Aux gas and Sheath gas were set to 72 and 14 units, respectively. An ionization reagent of 1.5 mM ammonium hydroxide was added at a rate of 0.05 mL/min through a Tee device using a secondary HPLC pump before the API chamber. Trolox was used as a standard for calibrating the system.

Characterization of Oxidized Products of α-Tocopherol Induced by AAPH. A mixture of 0.5 mL of 20 mM α-tocopherol (prepared in 7% RMCD) and 0.5 mL of 640 mM AAPH was incubated at 37 °C for 30 min and was extracted with 10 mL of methylene chloride. The methylene chloride phase was separated and washed with 5 mL of deionized water 3 times. The oxidized products of α-tocopherol in the methylene chloride phase were then analyzed by liquid chromatography/mass spectroscopy (LC/MS). With the exception of 100% acetonitrile as the mobile phase, the LC/MS conditions are the same as above.

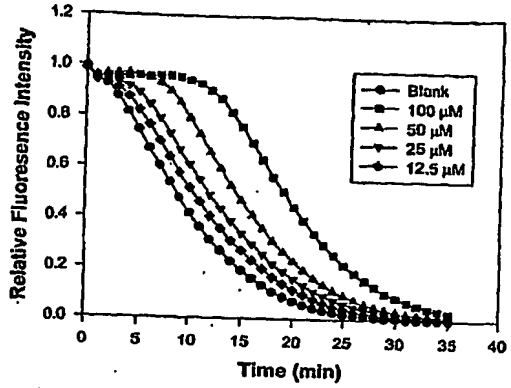

Figure 1. FL fluorescence decay curves induced by AAPH in the presence of α-tocopherol at different concentrations.

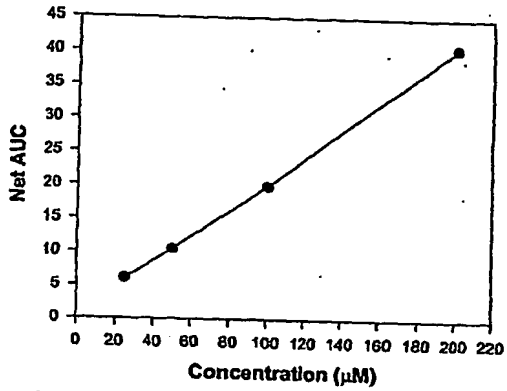

Figure 2. Regression of net AUC of α-tocopherol on different concentrations of α-tocopherol. The net AUC = $AUC_{sample} - AUC_{blank}$; the AUC was calculated by the equation previously described by Ou et al. (see *5*).

Photobleaching Experiment of BODIPY 581/591 C11. BODIPY 581/591 C11 was dissolved in a 1:9 butyronitrile and octane mixture (v/v) to give a 7.9×10^{-7} M solution; 200 μL of the BODIPY solution was then added into a 96 well polypropylene plate. The photostability of BODIPY was evaluated by monitoring the change of fluorescence intensity over a 1 h period. The fluorescence reading was taken every minute by a Bio-Tek fluorescence microplate reader FL600 (Bio-Tek, Winooski, VT) equipped with an excitation filter for 545/40 nm and an emission filter for 620/40 nm.

RESULTS

Method Validation. *(a) Linearity.* The correlation between the net area under the curve (AUC) for the antioxidant and its concentration was evaluated using four pure compounds. Figure 1 illustrates the FL fluorescence decay curves in the presence of α-tocopherol and AAPH. Figure 2 depicts the linear response between the concentration and the net AUC for α-tocopherol. Table 1 summarizes the net AUCs corresponding to the different concentrations and the linear coefficient (r^2) for the pure compounds.

(b) Limit of Quantitation (LOQ) and Limit of Detection (LOD). The LOQ is the lowest concentration on the calibration curve, while the LOD is the lowest amount of antioxidant that

Table 1. Net AUC vs Concentration[a]

compd	concn (μM)	net area	r^2
γ-oryzanol	25	28.94	
	12.5	15.87	0.9979
	6.25	8.51	
	3.125	4.32	
γ-tocopherol	100	28.45	
	50	14.83	0.9971
	25	7.78	
δ-tocopherol	75	36.11	
	50	27.52	
	25	15.56	0.9668
	12.5	8.34	
	6.25	4.46	
α-tocopherol	200	40.67	
	100	19.89	
	50	10.45	0.9990
	25	6.07	

[a] Regression equation is expressed as Y (net area) = kX (concentration) + intercept.

Table 2. Precision and Accuracy of the $ORAC_{FL-LIPO}$ Assay

vitamin E	QC1	QC2	QC3
nominal concn (μM)	40	80	160
	Run 1		
intramean (μM)	41.69	84.48	175.40
SD[a]	2.87	2.12	5.67
% RSD[b]	8.79	2.52	3.23
% REC[c]	104.25	105.60	109.62
N	4	4	4
	Run 2		
intramean (μM)	42.74	92.01	171.24
SD	2.65	5.12	10.86
% RSD	6.20	5.57	6.34
% REC	106.86	115.01	107.03
N	4	4	4
	Run 3		
intramean	39.0425	85.8525	167.21
SD	5.46	6.54	2.61
% RSD	13.99	7.62	1.56
% REC	97.60	107.31	104.50
N	4	4	4
	pooled runs		
intermean (μM)	38.16	87.45	171.28
SD	3.66	6.89	6.38
% RSD	9.66	5.24	3.71
% REC	95.39	109.31	107.05
N	12	12	12

[a] Standard deviation. [b] Relative standard deviation. [c] Recovery.

can be detected. In our experiment, the LOQ and LOD were determined to be 12.5 and 5.0 μM, respectively.

(c) Precision and Accuracy. Table 2 summarizes the precision and accuracy of the ORAC assay using α-tocopherol as a candidate compound. The precision, which is expressed as relative standard deviation (%RSD) for all quality control concentrations, was within ±15%. The accuracy of the method varies from 97.60 to 115.01% within individual batches and from 95.39 to 107.05% between all of the batches.

(d) Ruggedness. To determine the reproducibility of the method, a ruggedness experiment was performed. Using two COBAS FARA II analyzers, 12.5 μM γ-oryzanol was analyzed for 50 days. Results are shown in Figure 3.

ORAC Values for Pure Lipophilic Antioxidants. Table 3 lists the results for common lipophilic phenolic antioxidants, which were referenced as the TEs. γ-Oryzanol possesses the highest value of 3.0, while vitamin E acetate shows no antioxidant activity.

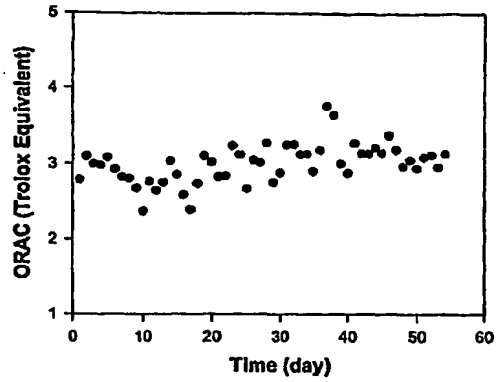

Figure 3. Ruggedness of the $ORAC_{FL-LIPO}$ assay. An amount of 12.5 μM γ-oryzanol was analyzed using two COBAS FARA II analyzers for 55 days.

Table 3. Relative ORAC Values of Pure Compounds ($n \geq 4$)

compds	ORAC
Trolox	1.0
α-tocopherol	0.50 ± 0.02
α-tocopherol acetate	0.00
(+)-γ-tocopherol	0.74 ± 0.03
(+)-δ-tocopherol	1.36 ± 0.14
nutriene (tocotrienols)	0.91 ± 0.04
2,2,5,7,8-pentamethyl-6-chromanol	1.02 ± 0.11
2-*tert*-butyl-4-methylphenol	0.41 ± 0.01
BHT	0.16 ± 0.01
γ-oryzanol	3.00 ± 0.26

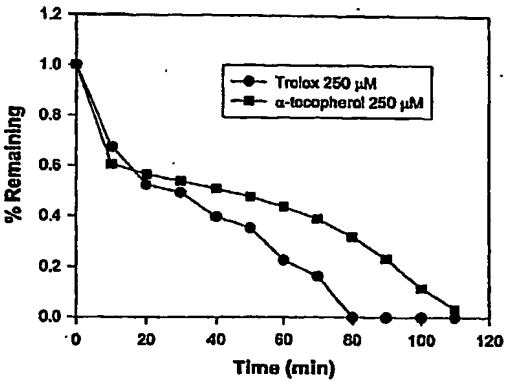

Figure 4. Kinetic curves for Trolox and α-tocopherol in the presence of AAPH. The reaction mixture contains 0.25 mM Trolox, 0.25 mM α-tocopherol, and 12.8 mM AAPH in RMCD (7% w/v) solution. The mixture was incubated at 37 °C in a HPLC autosampler, and 5 μL mixture was analyzed by HPLC at every 10 min interval until the reaction was completed.

Competitive Reaction between Trolox and α-Tocopherol with AAPH. The competitive reaction between Trolox and α-tocopherol with AAPH was examined, and the kinetic curves for Trolox and α-tocopherol were illustrated in Figure 4. It is evident that the reaction rate of Trolox with AAPH is much faster than that of α-tocopherol with AAPH.

Mechanistic Studies. The mechanisms for peroxidation of Trolox and α-tocopherol can be elucidated based on their

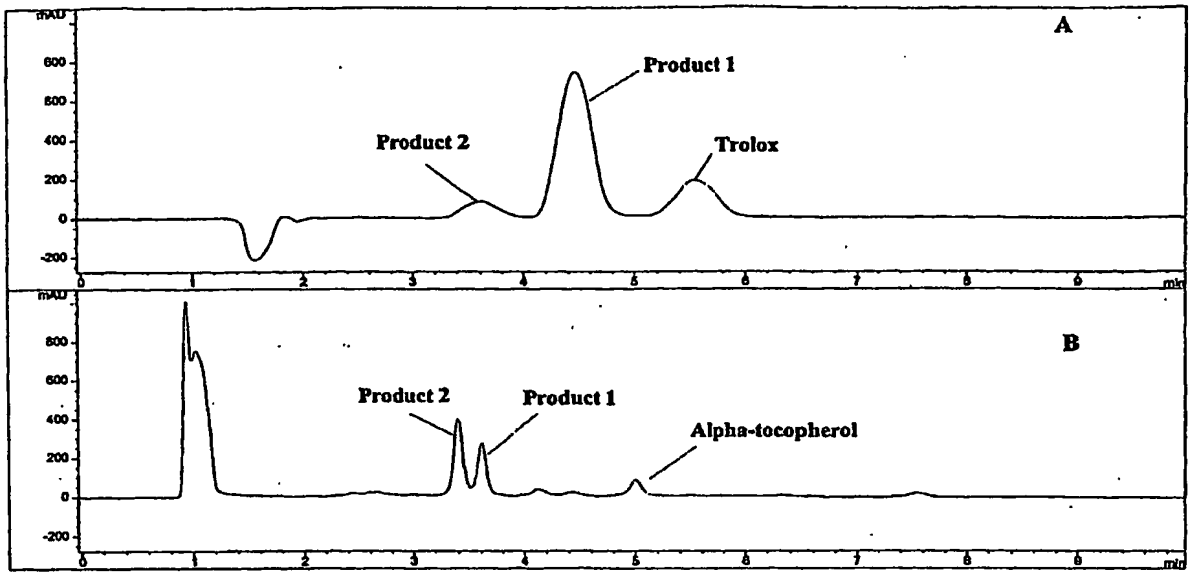

Figure 5. (a) HPLC chromatogram for the Trolox oxidation profile induced by AAPH. The mixture contained 0.4 mM Trolox and 512 mM AAPH in 75 mM phosphate buffer (pH 7.4) and was incubated at 37 °C for 30 min. The reaction products were separated by a Zorbax C18 column (2.1 mm × 150 mm, 3 μm) at 37 °C with a mobile phase of 70% methanol at a flow rate of 0.3 mL/min, and the UV detector was set at 280 nm. The oxidized products were characterized by using a Finnigan LCQ ion trap mass spectrometer equipped with an API chamber and an ESI source. (b) HPLC chromatogram for the α-tocopherol oxidation profile induced by AAPH. A mixture of 0.5 mL of 20 mM α-tocopherol (prepared in 7% RMCD) and 0.5 mL of 640 mM AAPH was incubated at 37 °C for 30 min and was extracted with 10 mL of methylene chloride. The methylene chloride phase was separated and washed with 5 mL of DI water 3 times. The oxidized products of α-tocopherol in the methylene chloride phase were then analyzed by LC/MS. With the exception of 100% acetonitrile as the mobile phase, the LC/MS conditions are the same as a.

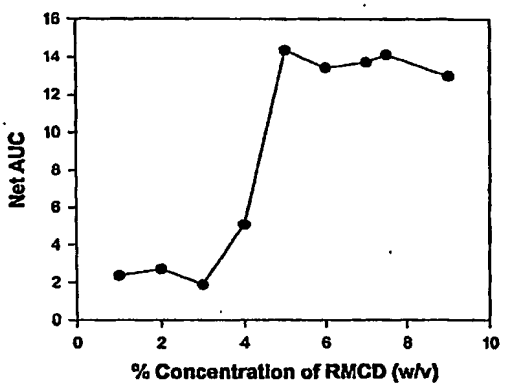

Figure 6. Effect of RMCD concentration (w/v) on the net AUC of 50 μM α-tocopherol.

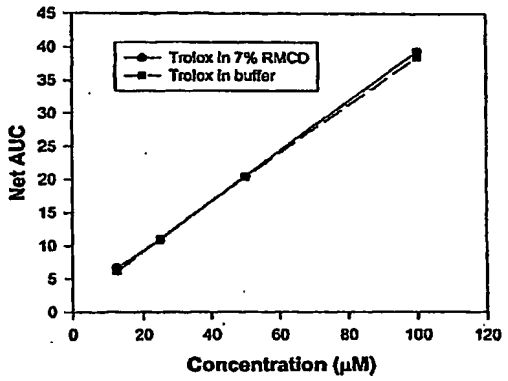

Figure 7. Regression of net AUC of Trolox on different concentrations of Trolox in the presence/absence of RMCD (7% w/v). Each data point represents an average of six separate measurements.

oxidized products obtained by LC/MS. As shown in Figure 5a,b, both Trolox and α-tocopherol were oxidized into two major products with 16 and 32 mass units increase. Figure 8 illustrates the proposed structures for oxidized products and the oxidation mechanism.

DISCUSSIONS

The ORAC assay was originally developed by Cao and coworkers (*6*) and was significantly improved by Ou et al. (*5*). However, the improved ORAC assay does not address the issue of lipophilic antioxidants because the assay is performed in aqueous solution. The application of CD for solubilization of lipophilic compounds has been extensively studied, particularly with the carotene/fatty acids−CD interaction (*3*, *4*, *7*). CDs are a group of naturally occurring cage molecules, which are built up from α-D-glucose units. Depending on the number of glucose moieties in the ring (6, 7, or 8), they are named α-, β-, and γ-cyclodextrin. CDs are doughnut-shaped and can bind a wide variety of organic "guest" compounds inside their apolar cavities in aqueous solution. The main driving force for this binding is hydrophobic interactions. Recently, Szente et al. studied the solubilization of carotene and fatty acids in aqueous solution, and their results indicated that the solubilizing power of CD derivatives are methylated-β-cyclodextrin ≫ hydroxypropylated-β-cyclodextrin = branched-β-cyclodextrin (*4*). The use of 10−40% methylated-β-cyclodextrin results in the 1000-fold enhancements of the aqueous solubility of lipophilic compounds. In light of Szente's work, we employed RMCD in the ORAC

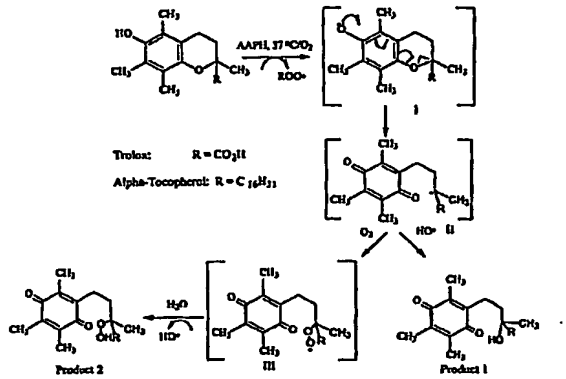

Figure 8. Proposed oxidation mechanism for Trolox and α-tocopherol in the presence of AAPH.

assay for lipophilic antioxidants. As demonstrated in the following sections, RMCD was determined to be an ideal solubility enhancer for the studied antioxidants, and for the first time, the antioxidant values for tocopherols, tocotrienols, and other common lipophilic antioxidants were obtained using the ORAC assay.

Effect of RMCD Concentration on the Solubility of Vitamin E in Aqueous Solution. Figure 6 shows the net AUC of 50 μM α-tocopherol at different RMCD concentrations (w/v) in 75 mM phosphate buffer solution (pH 7.4). The net AUC of α-tocopherol increases with the increase of RMCD concentration and reaches a plateau after 4% RMCD. The plateau indicates that α-tocopherol is completely soluble in 75 mM phosphate buffer solution. This conclusion was further confirmed by the HPLC studies (data not shown). Taking into account the concentration variations, 7% RMCD in 50% acetone solution was chosen for sample preparation.

Effect of RMCD on the ORAC Value of Trolox. Lipophilic antioxidants, such as the vitamin E family and oryzanol, consist of a long aliphatic tail (≥16 carbons) and a hydrophilic phenol group on the head. The tail can fit into a CD cavity allowing the hydrophilic phenol group to remain in aqueous solution. Therefore, the reactivity of the headgroup is not inhibited by CD complexation. RMCD itself consists of hydroxyl and methoxyl functional groups and is doughnut-shaped with an open moiety; therefore, RMCD does not possess any antioxidant activity nor does it prevent the complexed antioxidant molecule from functioning as antioxidant. To confirm this, the ORAC values of Trolox were examined in the presence (or absence) of 7% RMCD. Figure 7 shows the linear curves of the net AUC against the Trolox concentrations in phosphate buffer and 7% RMCD solution, respectively. It becomes obvious that the two curves are almost superimposed, suggesting that RMCD is inert in the ORAC assay.

Difference in ORAC Value between α-Tocopherol and Trolox. Because of the structural similarity, Trolox is expected to possess a similar ORAC value to that of α-tocopherol. However, our results reveal that the ORAC value of α-tocopherol is about 50% less than that of Trolox. The difference in ORAC value prompted us to investigate the reaction kinetics and mechanisms of Trolox/α-tocopherol with AAPH. Figure 4 shows the competitive reaction kinetic curves between Trolox and α-tocopherol in the presence of AAPH. As shown, the rate of Trolox with AAPH tends to be much faster than that of α-tocopherol with AAPH. This observation is in agreement with their ORAC values. At this point, we suggest that the inductive effect on the phenol group derived from the long ($C_{16}H_{31}$) aliphatic tail of α-tocopherol, an electron-donating group, may be the cause of the lower ORAC value. A remotely related analogue is that the higher alkanoic acid has lower acidity than that of acetic acid, because the alkyl groups manifest a small but significant electron donation to the carboxyl carbon (*8*). We further measured the ORAC value for α-tocopherol acetate, a popular ingredient in vitamin E supplements. Our result indicated that α-tocopherol acetate does not possess any antioxidant capacity under current experimental conditions. This result provides additional evidence to support our conclusion that the phenol group is an essential group for radical trapping antioxidant activity.

Mechanistic Studies on the Vitamin E Antioxidants. We examined the oxidized products of α-tocopherol and Trolox with AAPH by LC/MS and identified two oxidized products for each compound. Figure 5a,b shows the oxidation profiles for Trolox and α-tocopherol induced by AAPH obtained by HPLC, respectively. Figure 8 illustrates the proposed reaction mechanisms for Trolox and α-tocopherol in the presence of AAPH. As shown, the reaction was initiated by the formation of phenoxyl radical I due to a hydrogen atom being abstracted from the phenol group by the peroxyl radical. Phenoxyl radical I can further undergo intramolecular arrangement to form intermediate II, a tertiary carbyl radical. In the presence of O_2, intermediate II is peroxidized to yield intermediate III, a peroxyl radical that may abstract a hydrogen from the water molecule to yield product 2 and a highly reactive hydroxyl radical (HO•). Meanwhile, the intermediate II can couple with the generated HO• to produce product 1.

Structure–Activity Relationship. Table 3 summarizes the ORAC values for some common lipophilic antioxidants. The ORAC values for the three tocopherols are significantly different. The ORAC values of α-, γ-, and δ-tocopherol are 0.50, 0.74, and 1.36, respectively. The difference in ORAC value can be attributed to the steric effect of the ortho methyl groups. The number of methyl groups ortho to the phenol group of tocopherol decreases from two (α), one (γ), and zero (δ). A similar trend was observed for 2-*tert*-butyl-4-methylphenol (0.41 ± 0.01) and 2,6-di-*tert*-butyl-4-methylphenol (BHT) (0.16 ± 0.01). Apparently, the steric factor has a significant impact on the antioxidant activity; the less steric hindrance results in higher ORAC values. Our findings, however, are in contrast with the theoretical calculations by Wright and co-workers who calculated the O–H bond dissociation energies (BDE) for α- (75.78 kcal/mol), γ- (79.57 kcal/mol), and δ-tocopherol (81.43 kcal/mol) (*9*). On the basis of the BDE, they suggested that α-tocopherol has the highest antioxidant activity among the three. In fact, the ORAC value is a kinetic parameter while BDE relates to thermodynamics of a reaction. Therefore, BDE and ORAC values may not necessarily have the same trend. From our results, steric hindrance plays a significant negative role in antioxidant activity. γ-Oryzanol was found to possess a much higher ORAC value than that of any vitamin E antioxidant. In support of our result, a recent study by Godber and co-worker found that γ-oryzanol has a much higher antioxidant activity than tocopherols in protecting a cholesterol oxidation system accelerated by AAPH (*10*). The significantly greater ORAC value of γ-oryzanol can be attributed to the electronic contribution of an ortho methoxyl group and the larger π-conjugation system, involving 11 atoms, as compared to vitamin E with only 8 such atoms. A recent report of Mulder and co-workers on coenzyme $Q_{10}H_2$ reveals that the hydrogen atom abstraction is surprisingly easy from intramolecularly hydrogen-bonded meth-

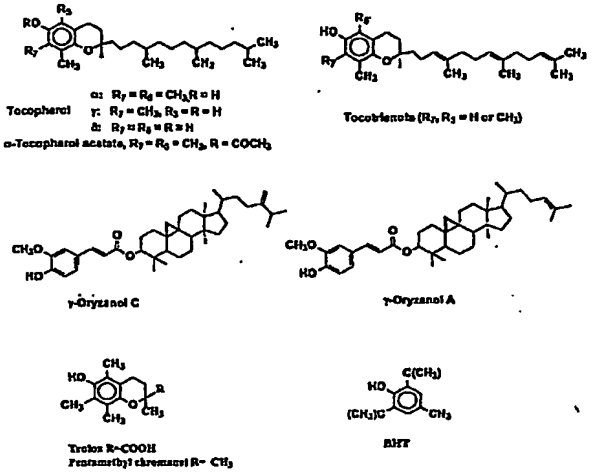

Figure 9. Structures of studied lipophilic antioxidants.

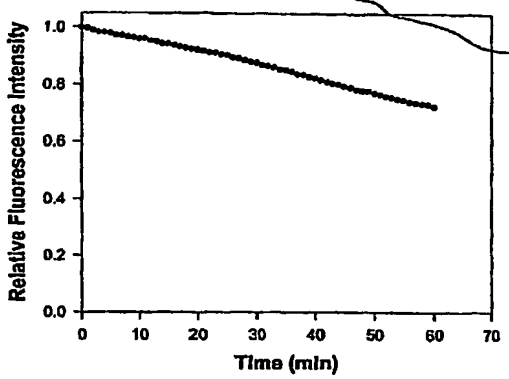

Figure 10. Fluorescence decay curve of BODIPY 581/591 C11 under microplate reader conditions (Bio-Tek FL 600). Solvent = butyronitrile/octane (1:9); temperature = 41 °C; concentration of BODIPY = 7.9×10^{-7} M.

oxyphenols (*11*). γ-Oryzanol possesses such a structural feature, and thus, it exhibits strong antioxidant activity. Figure 9 lists the molecular structures of lipophilic antioxidants studied in this paper.

Comparison of the $ORAC_{FL-LIPO}$ Assay with Other Related Methods. So far, only a few methods have been developed to measure lipophilic antioxidant activity. For example, Pulido et al. used a modified ferric-reducing/antioxidant power assay (FRAP) to measure the antioxidant activity of carotenoids (*12*); Acosta et al. modified the Trolox equivalent antioxidant capacity assay (TEAC) to measure the antioxidant activity of lipophilic vitamins (*13*). The drawbacks of using FRAP and TEAC for antioxidant activity measurement have been extensively discussed in our previous paper (*5*). Recently, Naguib developed a fluorometric method for lipophilic antioxidant activity, in which BODIPY 581/591 or BODIPY 665/676 was utilized as the fluorescent probe (*14*). Aldini et al. further modified Naguib's method to measure the lipid compartment of plasma (*15*). However, as shown in Figure 10, the BODIBY dye was found to be photobleached after it was exposed to excitation light. The photobleaching is likely caused by the cis/trans isomerization of the olefinic double bond in the presence of light (Figure 11). Therefore, the BODIBY-based method is not suitable for quantitation of antioxidant capacity.

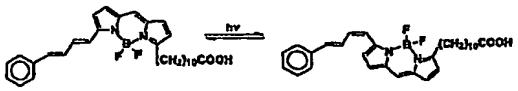

Figure 11. Possible photobleaching mechanism for BODIPY 581/591 after it was exposed to excitation light.

CONCLUSION

Using RMCD as the solubility enhancer, we successfully developed a validated ORAC assay for lipophilic antioxidants ($ORAC_{FL-LIPO}$). The $ORAC_{FL-LIPO}$ method was determined to be robust, reliable, and sensitive. We have also demonstrated that the phenol group is the key functional group for antioxidant activity, and the steric hindrance around the phenol group decreases ORAC values of tocopherols and other phenolic lipophilic antioxidants. The stereoelectronic effect derived from the long ($C_{16}H_{31}$) aliphatic tail of α-tocopherol also causes the lower ORAC value than that of Trolox. The oxidized Trolox and α-tocopherol have been identified using LC/MS, and the reaction mechanism was determined to follow a classic hydrogen atom transfer (HAT) mechanism. It is necessary to point out that the $ORAC_{FL-LIPO}$ assay does not measure the antioxidant activity of carotenoids and polyunsaturated fatty acids, since chemically carotenoids and fatty acids are not the chain-breaking antioxidants. Instead, they may act as the singlet oxygen scavengers and therefore follow a different reaction mechanism.

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LITERATURE CITED

(1) Halliwell, B. Antioxidants in human health and diseases. *Annu. Rev. Nutr.* 1996, *16*, 33−50.
(2) Burton, G. W.; Ingold, K. U. Vitamin E: application of the principles of physical organic chemistry to the exploration of its structure and function. *Acc. Chem. Res.* 1986, *19*, 194−201.
(3) Pfitzner, I.; Francz, P. I.; Biesalski, H. K. Carotenoid: methyl-beta-cyclodextrin formulations: an improved method for supplementation of cultured cells. *BBA Gen. Subjects* 2000, *1474*, 163−168.
(4) Szente, L.; Mikuni, K.; Hashimoto, H.; Szejtli, J. Stabilization and solubilization of lipophilic natural colorants with cyclodextrins. *J. Inclusion Phenom. Mol. Recognit. Chem.* 1998, *32*, 81−89.
(5) Ou, B.; Hampsh-Woodill, M.; Prior, R. Development and validation of an improved Oxygen Radical Absorption Capacity assay using fluorescein as a fluorescent Probe. *J. Agric. Food Chem.* 2001, *49*, 4619−4626.
(6) Cao, G.; Alessio, H. M.; Cutler, R. G. Oxygen-radical absorbance capacity assay for antioxidants *Free Radical Biol. Med.* 1993, *14*, 303−311.
(7) Szente, L.; Szejtli, J.; Szeman, J.; Kato, L. Fatty-acid cyclodextrin complexes − properties and applications *J. Inclusion Phenom. Mol. Recognit. Chem.* 1993, *16*, 339−354.
(8) Streitwieser, A.; Heathcock, C. H. Carboxylic Acids. In *Introduction to Organic Chemistry*; Macmillan Publishing Co., Inc.: New York, 1976; p 429.
(9) Wright, J. S.; Carpenter, D. J.; McKay, D. J.; Ingold, K. U. Theoretical calculation of substituent effects on the O−H bond strength of phenolic antioxidants related to vitamin E. *J. Am. Chem. Soc.* 1997, *119*, 4245−4252.
(10) Xu, Z.; Hua, N.; Godber, J. S. Antioxidant activity of tocopherols, tocotrienols, and γ-oryzanol components from rice bran against cholesterol oxidation accelerated by 2,2′-azobis(2-methylpropi-

onamidine) dihydrochloride. *J Agric. Food Chem.* 2001, *49*, 2077−2081.
(11) de Heer, M. I.; Mulder, P.; Korth, H.-G.; Ingold, K. U.; Lusztyk, J. Hydrogen atom abstraction kinetics from intramolecularly hydrogen bonded ubiquinol-10 and other (poly)methoxy phenols *J. Am. Chem. Soc.* 2000, *122*, 2355−2360.
(12) Pulido, R.; Bravo, L.; Saura-Calixo, F. Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/ antioxidant power assay. *J. Agric. Food Chem.* 2000, *48*, 3396−3402.
(13) Arnao, M. B.; Cano, A.; Acosta, M. The hydrophilic and lipophilic contribution to total antioxidant activity. *Food Chem.* 2001, *73*, 239−244.
(14) Naguib, Y. M. A. Antioxidant activities of astaxanthin and related carotenoids. *J. Agric. Food Chem.* 2000, *48*, 1150−1154.
(15) Aldini, G.; Yeum, K.−J.; Russell, R. M.; Krinsky, N. I. A method to measure the oxidizability of both the aqueous and lipid compartments of plasma *Free Radical Biol. Med.* 2001, *31*, 1043−1050.

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Analysis of Antioxidant Activities of Common Vegetables Employing Oxygen Radical Absorbance Capacity (ORAC) and Ferric Reducing Antioxidant Power (FRAP) Assays: A Comparative Study

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A total of 927 freeze-dried vegetable samples, including 111 white cabbages, 59 carrots, 51 snap beans, 57 cauliflower, 33 white onions, 48 purple onions, 130 broccoli, 169 tomatoes, 25 beets, 88 peas, 88 spinach, 18 red peppers, and 50 green peppers, were analyzed using the oxygen radical absorption capacity (ORAC) and ferric reducing antioxidant capacity (FRAP) methods. The data show that the ORAC and FRAP values of vegetable are not only dependent on species, but also highly dependent on geographical origin and harvest time. The two antioxidant assay methods, ORAC and FRAP, also give different antioxidant activity trends. The discrepancy is extensively discussed based on the chemistry principles upon which these methods are built, and it is concluded that the ORAC method is chemically more relevant to chain-breaking antioxidants activity, while the FRAP has some drawbacks such as interference, reaction kinetics, and quantitation methods. On the basis of the ORAC results, green pepper, spinach, purple onion, broccoli, beet, and cauliflower are the leading sources of antioxidant activities against the peroxyl radicals.

KEYWORDS: ORAC; FRAP; antioxidant activity; chain-breaking antioxidant; free radical; hydrogen atom transfer; single electron transfer

INTRODUCTION

There is now increasing interest in antioxidant activity of phytochemicals present in the diet. Antioxidants are believed to play a very important role in the body defense system against reactive oxygen species (ROS), which are the harmful byproducts generated during normal cell aerobic respiration (*1*). Increasing intake of dietary antioxidants may help to maintain an adequate antioxidant status and, therefore, the normal physiological function of a living system (*2*−*3*). Some functional foods and vegetables are the important sources of exogenous antioxidants. Their nutritional values are normally quantified by the total amount of certain components such as "total fat", "total calories", and "total carbohydrate" which are labeled in the nutrition facts sheet appearing on food packages. These indexes are intended to provide very useful nutritional information to consumers. Although antioxidants are recognized as important phytonutrients (*4*), currently there is no "total antioxidant" as a nutritional index available for food labeling because of the lack of standard quantitation methods. Unlike other nutrients, antioxidants are chemically diverse. The most common antioxidants present in vegetables are vitamins C and E, carotenoids, flavonoids, and thiol (SH) compounds, etc. The chemical diversity of antioxidants makes it difficult to separate and quantify individual antioxidants from the vegetable matrix. Therefore, it is desirable to establish a method that can measure the total antioxidant activity level directly from vegetable extracts. Recently, several methods have been developed to measure "total antioxidant activity" (*5*), "total antioxidant capacity" (*6*−*7*), or "total antioxidant potentials" (*8*−*9*). Among them, Trolox equivalent antioxidant capacity (TEAC) (*10*), total radical absorption potentials (TRAP) (*11*), ferric reducing/antioxidant power (FRAP) (*12*), and oxygen radical absorption capacity (ORAC) assays (*13*) are the representative ones. Mechanistically, these methods are based on either single electron transfer (SET) reaction or a hydrogen atom transfer (HAT) reaction between an oxidant and a free radical. For the SET-based methods (eq 1, M = metal ion), such as FRAP and TEAC, antioxidants are oxidized by oxidants, such as Fe (III) or $ABTS^{+\bullet}$ (eq 1). As a result, a single electron is transferred from the antioxidant molecule to the oxidant. The change of absorbance of either antioxidant or oxidant is measured by an ultraviolet−visible spectrometer and the absorbance value is used as the quantitation for the reducing capability of the antioxidant.

$$M(n) + A{-}H \rightarrow M(n-1) + A{-}H^{+} \quad (1)$$

$$ROO^{\bullet} + A{-}H \rightarrow ROOH + A^{\bullet} \quad (2)$$

$$ROO^{\bullet} + FL{-}H \rightarrow ROOH + FL^{\bullet} \quad (3)$$

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The HAT-based method (eqs 2 and 3, FL = fluorescent probe), such as ORAC and TRAP, utilizes a radical initiator to generate peroxyl radical ROO•. The ROO• abstracts a hydrogen atom from antioxidant preferably. As a result, the reaction between ROO• and the target molecule probe is retarded or inhibited. Because these chemically distinct methods are based on different reaction mechanisms, it is necessary to evaluate whether different methods can provide comparable antioxidant values for the same sample. In this paper, for the first time, the antioxidant activities of common vegetables, in a large sample size (total 927), from the U.S. market were analyzed using ORAC and FRAP procedures. Our results indicated that the two sets of results did not correlate well. This discrepancy has been extensively discussed from the mechanistic point of view.

MATERIALS AND METHODS

Chemicals. 2,4,6-tripyridyl-s-triazine (TPTZ) and $FeCl_3 \cdot 6H_2O$ were purchased from Sigma (St. Louis, MO). Fluorescein disodium (FL) and Trolox were obtained from Aldrich (Milwaukee, WI). 2,2′-azobis (2-amidino-propane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA (Richmond, VA).

Vegetable Samples. A total of 927 fresh vegetables were collected from various U.S. marketplaces at different harvesting seasons. They included 111 white cabbages, 59 carrots, 51 snap beans, 57 cauliflower, 33 white onions, 48 purple onions, 130 broccoli, 169 tomatoes, 25 beets, 88 peas, 88 spinach, 18 red peppers, and 50 green peppers. Freeze-drying was carried out for all the vegetables to remove the moisture. The freeze-dried vegetable samples were then packed in N_2-vacuumed amber bottles and stored in −80 °C before analysis.

Sample Preparation. Freeze-dried samples were accurately weighed into 0.5-g aliquots and 20 mL of acetone/water (50:50, v/v) extraction solvent was added. The mixture was shaken at 400 rpm at room temperature on an orbital shaker for an hour. The extracts were centrifuged at 14000 rpm for 15 min, and the supernatant was ready for analysis after appropriate dilution with 75 mM potassium phosphate buffer solution (pH 7.4).

Experimental Conditions. *ORAC Assay.* The automated ORAC assay was carried out on a COBAS FARA II spectrofluorometric centrifugal analyzer (Roche Diagnostic System Inc., Branchburg, NJ). The procedure was based on a previous report by Ou and co-workers (*14*). Trolox, a water-soluble analogue of vitamin E, was used as a control standard. The experiment was conducted at 37 °C under pH 7.4 condition with a blank sample in parallel. The analyzer was programmed to record the fluorescence of FL every minute after addition of AAPH. All fluorescent measurements are expressed relative to the initial reading. The final results were calculated using the differences of areas under the FL decay curves between the blank and a sample and were expressed as micromole Trolox equivalents (TE) per gram (μmol TE/g).

FRAP Assay. The FRAP assay was performed as previously described by Benzie and Strain (*12*), and was also carried out on a COBAS FARA II spectrofluorometric centrifugal analyzer (Roche). The experiment was conducted at 37 °C under pH 3.6 condition with a blank sample in parallel. In the FRAP assay, reductants ("antioxidants") in the sample reduce Fe (III)/tripyridyltriazine complex, present in stoichiometric excess, to the blue ferrous form, with an increase in absorbance at 593 nm. ΔA is proportional to the combined (total) ferric reducing/antioxidant power (FRAP value) of the antioxidants in the sample. The final results were expressed as micromole Trolox equivalents (TE) per gram on dried basis (μmol TE/g, db).

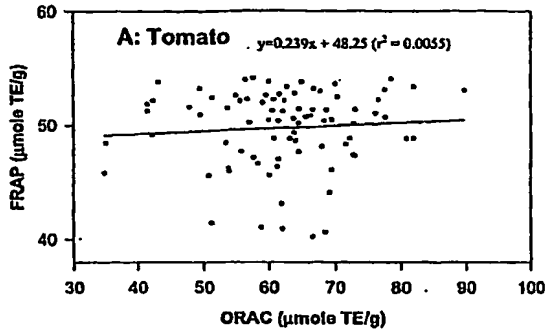
RESULTS

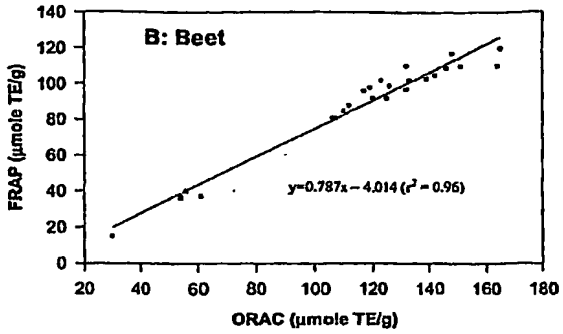
The vegetable extracts were analyzed by the standard FRAP and ORAC procedures, and the values obtained from the two methods were normalized to Trolox equivalents per gram on a freeze-dried basis. The distributions of the ORAC and FRAP values are illustrated in Figure 1A−M. The figures not only show cultivation dependency of the ORAC and FRAP values, but they also reveal the irregular relationship between ORAC and FRAP values. Table 1 summarizes the maximum, minimum, and median values of the antioxidant activities of the vegetable samples. Figures 2 and 3 show the antioxidant activity rank order of vegetables based on the data in Table 1.

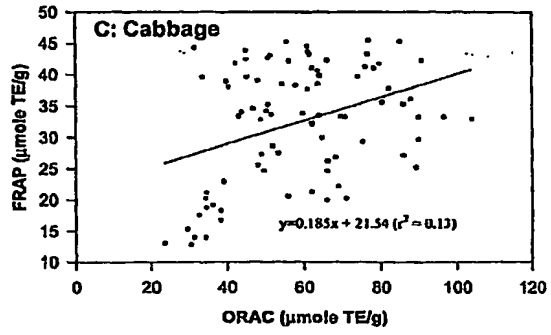
DISCUSSION

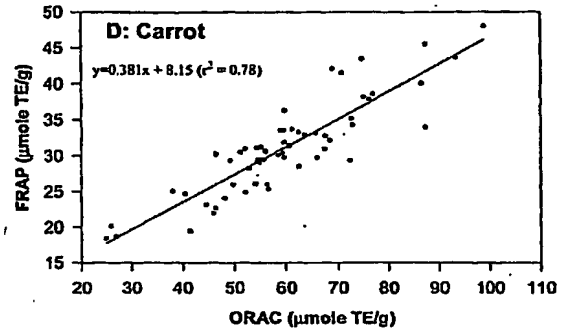
Cultivation Dependency of Antioxidant Capacity of Vegetables. Although we sampled the vegetables from various U.S. marketplaces without knowing the specific cultivation information for each vegetable species, from our results, one conspicuous conclusion is that the antioxidant activity of vegetables is highly dependent on cultivation conditions, as is reflected in Figure 1a−m. The antioxidant activity varies considerably from variety to variety. For instance, broccoli shows nearly 10-fold differences between highest and lowest values, green pepper exhibits almost 6-fold differences, and spinach reveals 2-fold differences. This large variability among the same vegetable can be apparently explained by the influences of different variety, location, and harvest season, etc., which would affect the level of antioxidants present in these vegetables. As reported by Prior et al., the major phytochemicals responsible for the antioxidant capacity most likely can be accounted for by the flavonoid compounds, which are known as secondary natural product metabolites (*15*). Apparently, the biosynthesis of these natural products is profoundly influenced by a number of factors, such as locations, weather conditions, and harvest periods, etc. Therefore, it is expected that the ORAC values vary accordingly. This similar phenomenon was observed for the FRAP group. Not only do vegetables show ORAC dependency on cultivar, but fruits also exhibit this same dependency. Prior and co-workers reported the ORAC values of fruit and leaf tissues of 87 highbush blueberries and the results show a wide range of ORAC values for different blueberry species (*16*). Another recent study has also shown that the antioxidant capacities of apples are dependent on cultivar (*17*). Currently, we are conducting further studies to characterize the major antioxidants present in these vegetables, and the outcomes will ultimately reveal the correlation between cultivation conditions and antioxidant activity from the chemistry point of view.

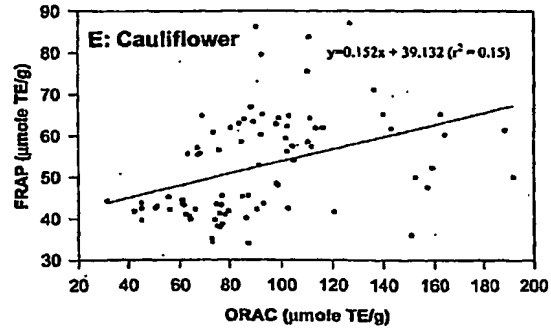
Antioxidant Activity Rank Order of the Vegetables. The comparative study of antioxidant activity is desirable not only from an academic point of view but also in the interest of vegetable producers and consumers. Consequently, there are plenty of papers attempting to rank antioxidant capacities of different plant extracts, including fruits and vegetables. For example, antioxidant activity of some vegetables based on ORAC results have been previously reported by Cao et al. (*18*). However, because of their limited sample size and drawbacks of the original ORAC method, previous ORAC values may not be representative in terms of antioxidant activity rank order (*14*). In the present study, for the first time, a large number of vegetables from various locations at different harvest seasons were evaluated using the ORAC and FRAP assays. To compare antioxidant activity on an equal basis, the moisture contained in the samples was removed by freeze-drying; thus, the results from this study provide us a fully comprehensive antioxidant activity profile of each examined vegetable. Figures 2 and 3 are the rank orders based on ORAC and FRAP values, respectively. Apparently, one cannot draw a clear conclusion on rank because the ORAC/FRAP data of different vegetables cover a broad range and overlap significantly among different vegetables, albeit the median values have some trend. For

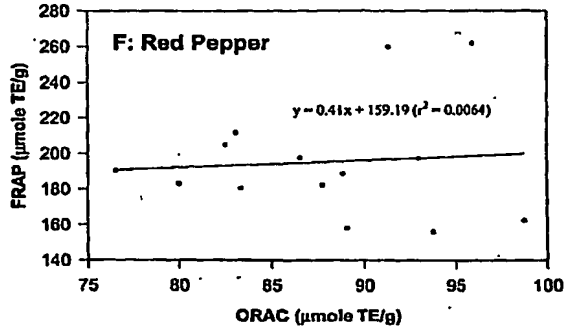

A: Tomato
y=0.239x + 48.25 (r² = 0.0055)
FRAP (μmole TE/g)
ORAC (μmole TE/g)

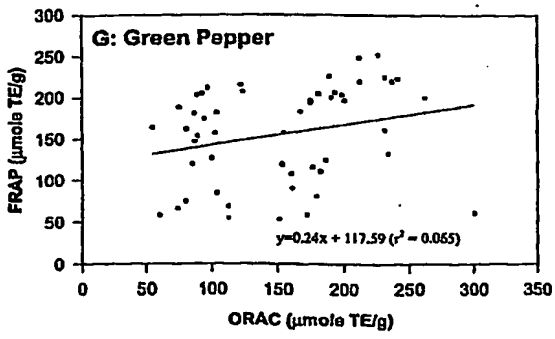

B: Beet
y=0.787x – 4.014 (r² = 0.96)
FRAP (μmole TE/g)
ORAC (μmole TE/g)

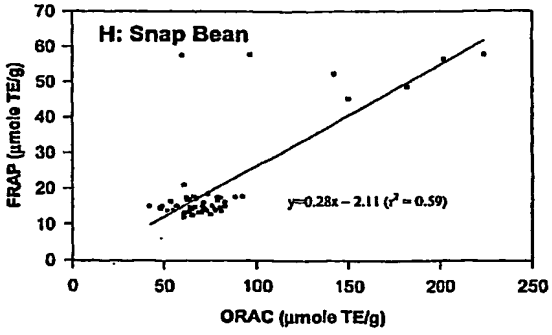

C: Cabbage
y=0.185x + 21.54 (r² = 0.13)
FRAP (μmole TE/g)
ORAC (μmole TE/g)


D: Carrot
y=0.381x + 8.15 (r² = 0.78)
FRAP (μmole TE/g)
ORAC (μmole TE/g)


E: Cauliflower
y=0.152x + 39.132 (r² = 0.15)
FRAP (μmole TE/g)
ORAC (μmole TE/g)


F: Red Pepper
y = 0.41x + 159.19 (r² = 0.0064)
FRAP (μmole TE/g)
ORAC (μmole TE/g)


G: Green Pepper
y=0.24x + 117.59 (r² = 0.055)
FRAP (μmole TE/g)
ORAC (μmole TE/g)


H: Snap Bean
y=0.28x – 2.11 (r² = 0.59)
FRAP (μmole TE/g)
ORAC (μmole TE/g)

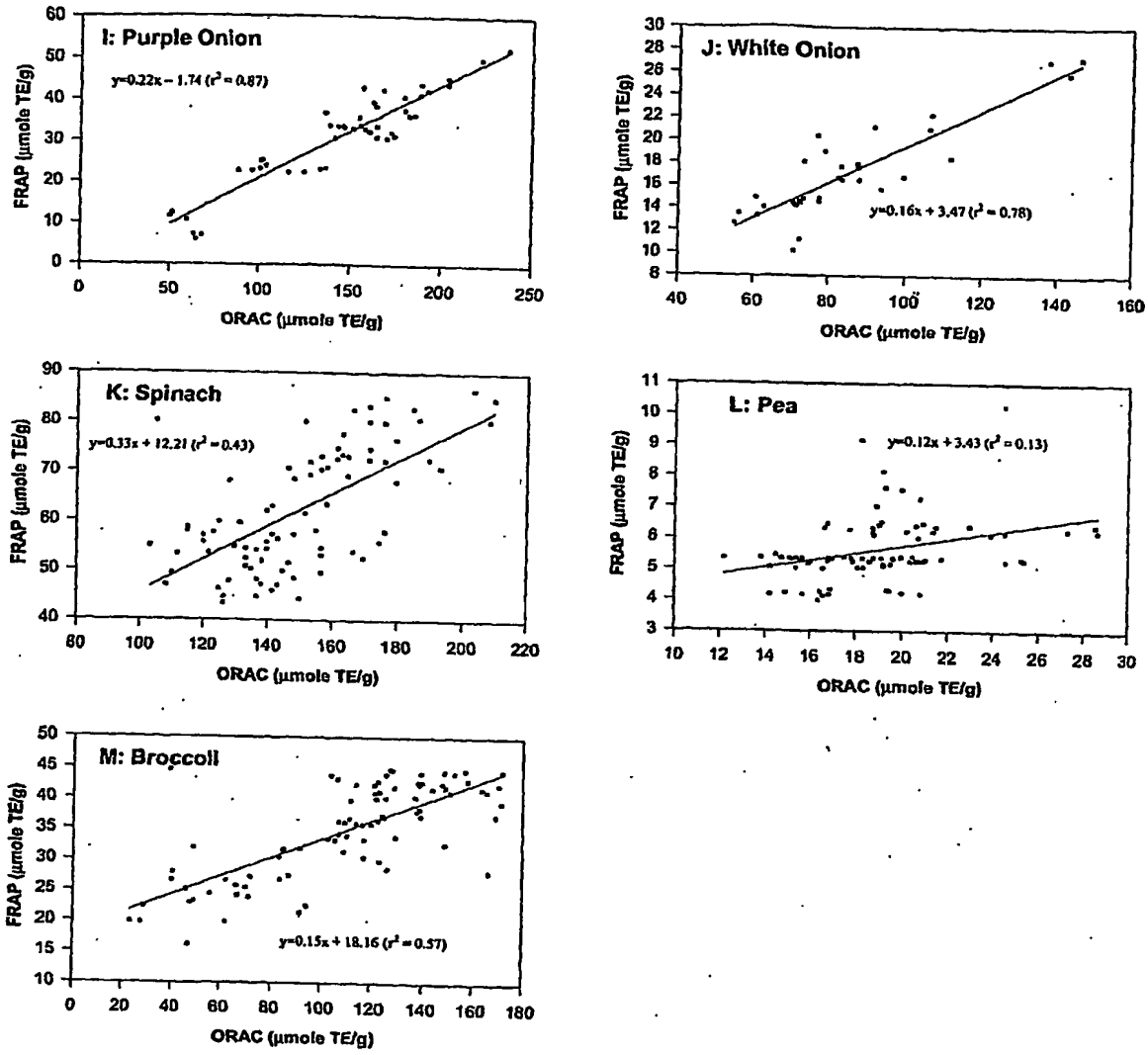


Figure 1. A–M. Correlation between the ORAC value and the FRAP value. The results are expressed as micromole Trolox equivalents per gram based on the freeze-dried weight (μmol TE/g, d.w.). With the exception of beet, carrot, purple onion, and white onion, there is no linear correlation between the ORAC value and the FRAP value.

Table 1. ORAC and FRAP Values of Vegetables (μmol TE/g) (n > 4)

		ORAC					FRAP				
species	sample size	max	min	median	mean	SD	max	min	median	mean	SD
pea	88	29	12	18	19	3	10	4	5	6	1
carrot	59	99	25	57	60	15	48	18	25	31	7
white cabbage	111	146	23	60	61	21	125	13	38	39	17
tomato	169	112	33	66	67	13	83	40	54	56	8
snap bean	51	223	42	70	79	37	58	12	15	20	13
white onion	33	146	55	78	85	23	27	10	16	17	4
red pepper	18	161	73	86	97	43	261	123	183	185	49
cauliflower	57	152	62	91	102	28	83	36	59	61	12
beet	25	165	30	120	115	36	120	12	96	86	29
broccoli	130	208	23	132	126	42	71	16	42	41	11
purple onion	48	237	50	153	143	46	52	6	33	31	11
spinach	88	234	103	148	152	26	94	43	58	64	13
green pepper	50	300	54	160	154	60	251	161	53	157	58

example, from a statistical point of view, the ORAC values among green pepper, spinach, and purple onion are the same. Therefore, for the rank to make sense, one has to specify the vegetable origin, harvest time, and data acquisition procedures.

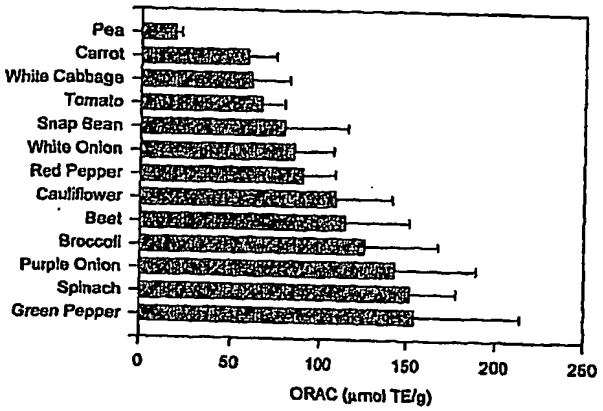


Figure 2. Rank order for antioxidant activities of common vegetables based on the ORAC values.

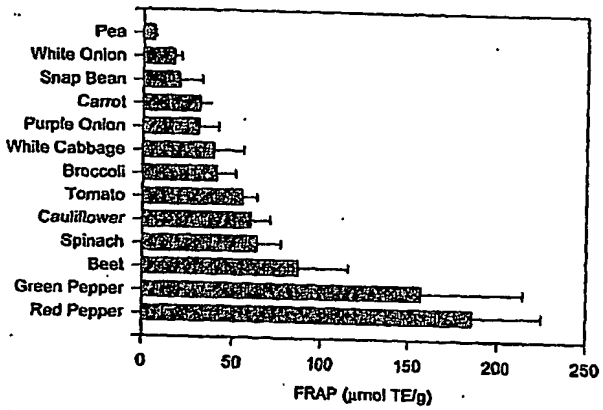


Figure 3. Rank order for ferric reducing capabilities of common vegetables based on the FRAP values.

Rank order based on the limited sample size and varieties is not representative and can result in misleading conclusions.

Antioxidant Reaction Mechanism. Both ORAC and FRAP values have been claimed to reflect total antioxidant activity (*19*–*20*). If this claim is valid, regardless of the different reaction mechanisms, their values should be comparable, and one should expect that the antioxidant active rank based on either ORAC or FRAP should have a similar trend. On the contrary, our data reveal that the FRAP and ORAC values do not correlate well. For example, the ratio of mean ORAC over FRAP value ranges from 0.52 to 5.00 for the twelve vegetables. The rank order based on the absolutely ORAC mean values is green pepper > spinach > purple onion > broccoli > beet > cauliflower > red pepper > white onion > snap bean > tomato > white cabbage > carrot > pea, whereas the rank based on the FRAP results is red pepper > green pepper > beet > spinach > cauliflower > tomato > broccoli > white cabbage > purple onion > carrot > snap bean > white onion > pea. Within vegetables, the plots of ORAC against FRAP (Figure 1a–m) also exhibit no trend for most vegetables, except for those of beet, onions, and carrots. The different results from the two assays warrant some discussions.

Herein, we attempted to analyze the two assays from the chemistry principles upon which they are based. In general, the antioxidants can be classified into two mechanistic categories: preventive antioxidants and chain-breaking antioxidants. Preventive antioxidants, such as superoxide dismutase, catalase, peroxidase, and transferrin, inhibit formation of reactive oxygen species. Chain-breaking antioxidants are compounds that scavenge oxygen radicals and thereby break radical chain sequences. They include vitamin C, vitamin E, uric acid, bilirubin, and polyphenols, et al. For chain-breaking antioxidants, there are two possible pathways in which antioxidants can play a role. The first pathway involves a hydrogen atom transfer (HAT), where the oxygen radical abstracts a hydrogen from the antioxidant, resulting in formation of a stable antioxidant radical. The following equations illustrate the stepwise process of HAT. We use an azo compound as a representative radical generator and LP–H as the lipid substrate:

$$R{-}N{=}N{-}R \rightarrow 2\,R^{\bullet} + N_2 \quad (4)$$

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet} \quad (5)$$

$$ROO^{\bullet} + LP{-}H \rightarrow ROOH + LP^{\bullet} \quad (6)$$

$$LP^{\bullet} + O_2 \rightarrow LPOO^{\bullet} \quad (7)$$

$$LPOO^{\bullet} + LP{-}H \rightarrow LPOOH + LP^{\bullet} \quad (8)$$

As illustrated, once the peroxyl radical (ROO•) is generated, the chain reactions of eqs 5–7 start, and, as a consequence, lipid molecules (LP–H) would be oxidized to lipid peroxides (LP–OOH). In the presence of antioxidants (ArOH), the lipid peroxidation chain reaction can be interrupted as follows:

$$ROO^{\bullet} + ArOH \rightarrow ArO^{\bullet} + ROOH \quad (9)$$

For phenolic antioxidants, the formed phenoxyl radical ArO• is relatively stable, and it only reacts slowly with substrate LPH but rapidly with peroxyl radical ROO•. For example, α-tocopherol (α-TOH), known as the most effective lipid-soluble chain-breaking antioxidants in vivo, reacts with peroxyl radical with a rate constant of about $10^6\ M^{-1}s^{-1}$, which is much faster than the reaction of peroxyl radicals with lipid substrate, typically $10^1\ M^{-1}s^{-1}$. The second possible pathway in which antioxidant de-activative free radicals is single electron transfer (SET) as illustrated below:

$$ROO^{\bullet} + ArOH \rightarrow ROO^{-} + ArOH^{+\bullet} \quad (10)$$

$$ROO^{-} + ArOH^{+\bullet} \rightarrow ArO^{\bullet} + ROOH \quad (11)$$

The net result from above is the same as from the HAT mechanism. However, when compared to HAT, the SET mechanism is strong-solvent-dependent due to solvent stabilization of the charged species. The question raised here is which mechanism physiologically reflects the antioxidant preventive action. More recently, Wright and co-workers used a procedure based on density functional theory to calculate the gas-phase bond dissociation enthalpy (BDE) and ionization potential for molecules belonging to the class of phenolic antioxidants, including tocopherols, catechins, aminophenols, and stilbenes related to resveratrol. Their results demonstrated that in most cases HAT will be dominant (*21*). It is logical with the biological oxidation processes, in which an oxygen molecule is reduced to the final product H_2O with some degree of SET involvement (oxygen oxidation state changed from 0 to −2). More importantly, it is also a hydrogen atom transfer process because oxygen is hydrogenated and the reductants are dehydrogenated. For example, dihydroflavin oxidizing to flavins, hydroquinones oxidizing to quinones, and thiols oxidizing to disulfides. It is

clear that hydrogen atom transfer (HAT) reaction concurs with electron transfer reaction and plays a dominant role in biological redox reactions. Therefore, the ORAC principle is closely related to biological functions of chain-breaking antioxidants.

Principle of FRAP. The relevant chemical reaction of the FRAP method involves a single electron reaction between Fe $(TPTZ)_2$ (III) and a single electron donor ArOH.

$$Fe(TPTZ)_2(III) + ArOH \rightarrow Fe\ (TPTZ)_2(II) + ArOH^{+\bullet} \quad (12)$$

Benzie and co-workers (*12*) considered the antioxidant as any species that reduces the oxidizing species that would otherwise damage the substrates. And the authors further treat the "total antioxidant power" as the "total reducing power". The antioxidant activity is then interpreted as the reducing capability.

To accurately measure the total reducing power, the following conditions must be met. (1) All, and only, antioxidants can reduce Fe $(TPTZ)_2$(III) under the reaction conditions (thermodynamics). (2) The reaction rate must be sufficiently fast enough that the reaction can be completed in a short assay time (e.g., 4 min in the actual FRAP assay) (kinetics). (3) The oxidized antioxidant, $ArOH^{+\bullet}$, and its secondary reaction products should have no absorption at 593 nm, the maximum absorption of Fe $(TPTZ)_2$ (II).

In fact, these conditions are very difficult to meet. First, the standard redox potential of Fe(III)/Fe(II) is 0.77 V; any compound with lower redox potential can theoretically reduce Fe (III) to Fe (II) and contributes to the FRAP values resulting in falsely high FRAP values. Therefore, the reason for choosing Fe (III) as an oxidant seems to be too arbitrary. Second, not all antioxidants reduce Fe (III) at a fast rate as anticipated. For example, Pulido and co-workers (*22*) recently examined the FRAP assay of dietary polyphenols in water and methanol. The absorption (A_{593}) was slowly increasing even after several hours of reaction time. The polyphenols with such behavior include caffeic acid, tannic acid, ferulic acid, ascorbic acid, and quercetin, etc. Besides polyphenols, thiol compounds also react with Fe $(TPTZ)_2$(III) slowly. Our own experiment shows that the reaction of glutathione with Fe $(TPTZ)_2$(III) is a very slow reaction and the reducing power cannot be correctly measured (unpublished results). At this point, the FRAP reaction is too slow to be of any practical use. Third, another limitation of the FRAP assay is the possible interference due to the UV−Vis absorption at 593 nm by compounds other than Fe $(TPTZ)_2$ (II). For example, Benzie and co-workers (*12*) reported an unusually high FRAP value for bilirubin (twice that of Trolox and ascorbic acid). In fact, it is known that when bilirubin is oxidized, it is transformed to beliverdin which has a strong absorption at 593 nm ($\epsilon_{593} = 1 \times 10^4$). Therefore, the FRAP assay cannot be used in biological samples. Many vegetable extracts are colored and may have similar interference. Finally, the FRAP assay depends on the reduction of a ferric tripyridyltriazine Fe $(TPTZ)_2$(III) complex to the ferrous tripyridyltriazine Fe$(TPTZ)_2$(II) by an antioxidant at a low pH of 3.6. However, the low pH can significantly inhibit one electron transfer from the antioxidant to the ferric ion. FRAP results reflect only the antioxidant reducing potential based on ferric ion instead of the antioxidant preventive effect. Clearly, the FRAP assay actually measures the reducing capability based upon ferric ion, which is not relevant to antioxidant activity mechanistically and physiologically, let alone the total antioxidant capacity. On the basis of these facts, we feel that it is not appropriate to use the FRAP value as an indicator for "total antioxidant power".

Principle of the ORAC Assay. The ORAC assay was initially developed by Cao et al. and was significantly improved by Ou and co-workers (*14*). In the improved ORAC assay, fluorescein was the chosen fluorescent probe. Ou and co-workers have also identified the oxidized fluorescein products and the reaction mechanism was determined to follow the HAT mechanism. Under this reaction condition, one mole of AAPH loses a dinitrogen to generate two moles of AAPH radical at a constant rate (eq 4). In air-saturated solution, the generated AAPH radical reacts with O_2 rapidly (eq 5; $k_5 \sim 10^9$ $mol^{-1}\cdot s^{-1}$) to give a more stable peroxyl radical ROO$^\bullet$. The loss of fluorescence of fluorescein is an indication of the extent of damage from its reaction with the peroxyl radical. In the presence of antioxidant, ROO$^\bullet$ abstracts a hydrogen atom from the antioxidant to form hydroperoxide (ROOH) and a stable antioxidant radical (ArO$^\bullet$); as a result, the damage to fluorescein induced by peroxyl radical is inhibited. The protective effect of an antioxidant is measured by assessing the area under the fluorescence decay curve (AUC) of the sample compared to that of the blank in which no antioxidant is present. Ou and co-workers have shown that under the ORAC experimental conditions fluorescence decrease is independent of concentrations of FL but first order with AAPH concentration. Thus, the reaction rate is limited by eq 4 ($k_4 = 3.19 \times 10^{-7}$ $mol^{-1}\cdot s^{-1}$). Most of the samples do not affect the thermo-decomposition rate of AAPH, and AAPH itself does not react directly with the sample. As a result, the ORAC assay directly measures the antioxidant activities of chain-breaking antioxidants against peroxyl radicals. Therefore, we suggest that ORAC values be used as a guideline for "peroxyl radical absorption capacity" of vegetables.

In summary, the antioxidant activities of 927 vegetables have been measured by the ORAC and FRAP assays. On the basis of our knowledge of antioxidant chemistry, it is concluded that the ORAC values reflect the peroxyl radical scavenging activity of vegetables. In contrast, the FRAP assay estimates only the Fe (III) reducing activity, which is not necessarily relevant to antioxidant activity physiologically and mechanistically. Thus, we suggest that the antioxidant rank order should be based on ORAC results. To our best knowledge, the study reported here is the most comprehensive antioxidant study on the common vegetables so far, in which nearly 1,000 vegetable extracts were analyzed. Hence, our results are representative and provide some valuable data for establishment of recommended antioxidant daily allowance in the future. However, the ORAC assay is not a "total antioxidant activity assay", because it only measures antioxidant activity against peroxyl radicals. Biologically relevant reactive oxygen species (ROS) also include $O_2^{-\bullet}$, HO$^\bullet$, ONOO−, and singlet oxygen. As different ROS have different reaction mechanisms, to completely evaluate antioxidant activity is a rather difficult task without a short-cut, and using one assay result to claim "total antioxidant activity" is oversimplified and thus inappropriate. To elucidate a full profile of antioxidant activity against various ROS, comprehensive assays are needed.

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ABBREVIATIONS USED

Trolox, 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid; AAPH, 2,2′-azobis (2-amidino-propane) dihydrochloride; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate); ORAC, oxygen radical absorbance capacity; HAT, hydrogen atom transfer;

TEAC, Trolox equivalent antioxidant capacity; FRAP, ferric reducing antioxidant power.

LITERATURE CITED

(1) Gutteridge, J. M. C.; Halliwell, B. Free radicals and antioxidants in the year 2000 − A historical look to the future. *Ann. N. Y. Acad. Sci.* **2000**, *899*, 136−147.

(2) Kaur, C.; Kapoor, H. C. Antioxidants in fruits and vegetables − the millennium's health. *Int. J. Food Sci. Technol.* **2001**, *36*, 703−725.

(3) Record, I. R.; Dreosti, I. E.; McInerney, J. K. Changes in plasma antioxidant status following consumption of diets high or low in fruit and vegetables or following dietary supplementation with an antioxidant mixture. *Brit. J. Nutr.* **2001**, *85*, 459−464.

(4) Panel on Dietary Antioxidants and Related Compounds, Subcommittees on Upper Reference Levels of Nutrients and Interpretation and Uses of DRIs, Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, Food and Nutrition Board. *Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids*; National Academy Press: Washington, DC, 2000; p 529.

(5) Rice-Evans, C. A. Measurement of total antioxidant activity as a marker of antioxidant status in vivo: Procedures and limitations. *Free Radical Res.* **2000**, *33*, S59−S66.

(6) Young, I. S. Measurement of total antioxidant capacity. *J. Clin. Pathol.* **2001**, *54*, 339−339.

(7) Kirschbaum, B. Total urine antioxidant capacity. *Clin. Chim. Acta* **2001**, *305*, 167−173.

(8) Lissi, E.; Salimhanna, M.; Pascual, C.; Delcastillo, M. D. Evaluation of total antioxidant potential (trap) and total antioxidant reactivity from luminol-enhanced chemiluminescence measurements. *Free Radical Biol. Med.* **1995**, *18*, 153−158.

(9) Simonetti, P.; Pietta, P.; Testolin, G.; Polyphenol content and total antioxidant potential of selected Italian wines. *J. Agric. Food Chem.* **1997**, *45*, 1152−1155.

(10) van den Berg, R.; Haenen, G. R. M. M.; van den Berg, H. Applicability of an improved Trolox equivalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurements of mixtures. *Food Chem.* **1999**, *66*, 511−517.

(11) Evelson, P.; Travacio, M.; Repetto, M. Evaluation of total reactive antioxidant potential (TRAP) of tissue homogenates and their cytosols. *Arch. Biochem. Biophys.* **2001**, *388*, 261−266.

(12) Benzie, I. F. F.; Strain, J. J. Ferric reducing antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Methods Enzymol.* **1999**, *299*, 15−27.

(13) Cao, G. H.; Prior, R. L. Measurement of oxygen radical absorbance capacity in biological samples. *Methods Enzymol.* **1999**, *299*, 50−62.

(14) Ou, B.; Hampsch-Woodill, M.; Prior, R. L. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *J. Agric. Food Chem.* **2001**, *49*, 4619−4626.

(15) Prior, R. L.; Cao, G. H.; Martin, A.; Sofic, E.; McEwen, J.; O'Brien, C.; Lischner, N.; Ehlenfeldt, M.; Kalt, W.; Krewer, G.; Mainland, C. M. Antioxidant capacity as influenced by total phenolic and anthocyanin content, maturity, and variety of *Vaccinium* species. *J. Agric. Food Chem.* **1998**, *46*, 2686−2693.

(16) Ehlenfeldt, M. K.; Prior, R. L. Oxygen radical absorbance capacity (ORAC) and phenolic and anthocyanin concentrations in fruit and leaf tissues of highbush blueberry. *J. Agric. Food Chem.* **2001**, *49*, 2222−2227.

(17) van der Sluis, A. A.; Dekker, M.; de Jager, A.; Jongen, W. M. F. Activity and concentration of polyphenolic antioxidants in apple: effect of cultivar, harvest year, and storage conditions. *J. Agric. Food Chem.* **2001**, *49* (8), 3606−3613.

(18) Cao, G. H.; Sofic, E.; Prior, R. L. Antioxidant capacity of tea and common vegetables. *J. Agric. Food Chem.* **1996**, *44*, 3426−3431.

(19) Wang, H.; Cao, G. H.; Prior, R. L. Total antioxidant capacity of fruits. *J. Agric. Food Chem.* **1996**, *44*, 701−705.

(20) Benzie, I. F. F.; Szeto, Y. T. Total antioxidant capacity of teas by the ferric reducing/antioxidant power assay. *J. Agric. Food Chem.* **1999**, *47*, 633−636.

(21) Wright, J. S.; Johnson, E. R.; Carpenter, D. J. Predicting the Activity of Phenolic Antioxidants: Theoretical method, analysis of substituent effects, and application to major families of antioxidants. *J. Am. Chem. Soc.* **2001**, *123*, 1173−1183.

(22) Pulido, R.; Bravo, L.; Saura-Calixo, F. Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. *J. Agric. Food Chem.* **2000**, *48*, 3396−3402.

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Novel Fluorometric Assay for Hydroxyl Radical Prevention Capacity Using Fluorescein as the Probe

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A novel fluorometricmethod has been developed to evaluate hydroxyl radical prevention capacity using fluorescein (FL) as the probe. The hydroxyl radical is generated by a Co(II)-mediated Fenton-like reaction, and the hydroxyl radical formation under the experimental condition is indirectly confirmed by the hydroxylation of *p*-hydroxybenzoic acid. The fluorescence decay curve of FL is monitored in the absence or presence of antioxidant, the area under the fluorescence decay curve (AUC) is integrated, and the net AUC, which is an index of the hydroxyl radical prevention capacity, is calculated by subtracting the AUC of the blank from that of the antioxidant. Gallic acid is chosen as a reference standard, and the activity of sample is expressed as gallic acid equivalents. The method is rigorously validated through linearity, precision, accuracy, and ruggedness. A wide range of phenolic antioxidants is analyzed, and the hydroxyl radical prevention capacity is mainly due to the metal-chelating capability of the compounds.

KEYWORDS: Assay; polyphenolics; hydroxyl radicals; preventive antioxidant; Fenton reaction

INTRODUCTION

Accumulated evidence indicates that reactive oxygen species (ROS), such as peroxyl radicals ($ROO^•$), hydroxyl radicals ($HO^•$), superoxide ion ($O_2^{-•}$), and singlet oxygen ($^1O_2$), are involved in the pathophysiology of aging and a multitude of diseases, such as cancer, Alzheimer's disease, and Parkinson's disease (*1, 2*). To counteract the damages of the ROS on living cells, a defense system is designed biologically to neutralize the ROS or to prevent the ROS from being generated in the first place. Depending on the reaction mechanisms, antioxidants are often classified into two major categories: radical chain-breaking antioxidants and preventive antioxidants (*3*). Chain-breaking antioxidants convert reactive free radicals (e.g., $HO^•$) to stable and thus nonaggressive molecules through hydrogen atom transfer reactions between $HO^•$ and the antioxidants. As a result, the autoxidation chain reactions between the free radicals and the cellular molecules are terminated. Preventive antioxidants inhibit the oxidation reaction from occurring by either converting the precursors of the ROS to unreactive species or inhibiting the oxidation reaction. On the basis of their molecular nature, the preventive antioxidants include enzymatic and nonenzymatic antioxidants. Well-known enzymatic antioxidants are superoxide dismutase (SOD), catalase, and glutathione peroxidase. Nonenzymatic antioxidants include oxidative enzyme inhibitors and metal chelators (L). Examples of the former include anti-inflammatory medicines such as ibuprofen, which inhibits the activity of cyclooxygenase. Metal chelators deactivate transition metals from reaction with hydrogen peroxide to form reactive oxygen species believed to be hydroxyl radicals. It is evident that the antioxidant defense "team" in living cells contains individual antioxidants that function in very different tasks in the battles against oxidative stress and ROS. Therefore, it is imperative that to comprehensively evaluate the antioxidant activity of food nutrients in vitro, we need a broad range of assays that can cover all aspects of antioxidant activity. It is impossible to have a one-fits-for-all assay. Although there is a validated assay for peroxyl radical absorbance capacity (ORAC) (*4, 5*), no such assay has been reported for hydroxyl radicals. This paper will address that issue.

One condition for hydroxyl radical generation in vitro and in vivo involves two essential components: oxidizable metal ions and hydrogen peroxide. The fact that the mixture of hydrogen peroxide and transition metals such as Fe(II) and Co(II) is a strongly oxidizing and hydroxylating reagent prompted the suggestion of hydroxyl radical involvement during the reaction. The exact mechanisms of Fenton-like reaction are extremely complex, and there is no conclusive answer for or against the involvement of hydroxyl radicals (*6*). The plain fact all have agreed upon is that the mixture of Fe(II)/Co(II) and H_2O_2 is an extremely powerful oxidant that is fatal to living cells, which have an antioxidant defense system to eliminate the possibility of reaction between H_2O_2 and metal ions (*7*).

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Removal of either of the two reactants will accomplish such a task. Catalase converts H_2O_2 to O_2 and H_2O, and metal chelators bind metal ions so that they become inert toward H_2O_2. Dietary nutrients contain metal chelators that will act as preventive antioxidants against the formation of hydroxyl radicals. Quantifying the activity of the phytochemicals in preventing hydroxyl formation in vitro will be a valuable guide to antioxidant clinical research. Herein we present a fluorometrically based assay for the metal-chelating capacity of polyphenolic compounds and a food matrix. For simplification purposes, we termed this new assay HORAC, standing for hydroxyl (HO) radical (R) averting (A) capacity (C).

MATERIALS AND METHODS

Chemicals and Apparatus. All flavonoid compounds and 30% hydrogen peroxide were purchased from Sigma (St. Louis, MO). Iron(II) fluoride, cobalt(II) fluoride tetrahydrate, picolinic acid (PA), gallic acid, 3,4-dihydroxybenzoic acid, *p*-hydroxybenzoic acid, and fluorescein disodium were obtained from Aldrich (Milwaukee, WI). Various fruit extracts were obtained in house. All analyses were performed on a COBAS FARA II analyzer (Roche Diagnostic System Inc., Branchburg, NJ; excitation wavelength = 493 nm and emission filter = 515 nm). Identification of the reaction product of *p*-hydroxybenzoic acid and CoF_2/PA−H_2O_2 was performed by an HP 1100 high-performance liquid chromatography system (Hewlett-Parkard, Palo Alto, CA) coupled with a Finnigan MAT LCQ ion trap mass spectroscopic detector (ThermoFinnigan, San Jose, CA): column, Zorbax (Hewlett-Packard) C18 (2.1 × 150 mm, 3 μm); mobile phase, 70% methanol; flow rate, 0.3 mL/min; UV detector at 280 nm.

Reagent Preparation. Fluorescein solution was prepared as follows: 0.0225 g of fluorescein disodium (70%) was dissolved in 50 mL of phosphate buffer and vortexed to homogeneity. The solution, denoted concentrated stock solution, was kept in a −80 °C freezer for future use. Fifty microliters of concentrated stock solution was diluted to 10 mL with phosphate buffer to yield the stock solution (4.19 μM); 0.24 mL of stock solution was further diluted to 15 mL with phosphate to give a working solution with a fluorescein concentration of 6.7×10^{-2} μM. A 1.1 M hydrogen peroxide solution was prepared by diluting 30% H_2O_2 solution (8.8 M) with distilled water. A cobalt solution was prepared by dissolving 15.7 mg of cobalt(II) fluoride tetrahydrate and 20 mg of picolinic acid in 10 mL of distilled water.

Sample Preparation. Gallic acid, flavonoids, and phenolic acids (see Table 4 for individual compounds) were dissolved in methanol and diluted with pH 7.4 phosphate buffer (75 mM) for analysis. The fruit samples were initially ground in a mechanical mill to produce a fine powder; then 0.5 g was accurately weighed, and 20 mL of acetone/water (50:50, v/v) was added. The mixture was shaken at 400 rpm at room temperature on an orbital shaker for 1 h. The extracts were centrifuged at 14000 rpm for 15 min, and the supernatant was ready for analysis after appropriate dilution with phosphate buffer solution. Fruit extracts (~0.2 g) were dissolved into 50% acetone and water for analysis. For liquid samples, a 20 mL aliquot of sample was centrifuged for 15 min, and the supernatant was ready for analysis after further dilution with phosphate buffer.

Hydroxyl (HO•) Radical Averting Capacity Assay. The COBAS FARA II was programmed to use a two-reagent system (reaction mode 3, P−I−SR1−I−SR2−A). The reaction mode pipetted and transferred the sample (20 μL) and main reagent (360 μL of FL, 8.61×10^{-8} M) into the main reagent wells of their respective cuvette rotor positions. The rotor spins and the reagents are mixed and incubated for 30 s. After the rotor stops spinning, a start reagent (SR1), 10 μL of hydrogen peroxide (1.1 M), is pipetted into the appropriate start reagent well in the curvette rotor. Next, the analyzer starts spinning, mixing the sample/FL with H_2O_2 for 30 s. The initial reading of fluorescence was taken. Finally, 10 μL of cobalt(II) fluoride solution ([Co] = 9.2 mM) was pipetted into the appropriate start reagent well in the curvette rotor. The analyzer then spins to mix the solutions, and the oxidative reaction starts. Hence, the sample makes up 5% of the reaction volume, and the final concentrations of FL, Co(II), and H_2O_2 are 0.062 μM, 230 μM, and 2.75×10^{-2} M, respectively. Between transfers, both sample and reagent transfer pipets are washed with cleaning solution to eliminate sample cross-contamination. Fluorescence readings are taken at 0.5 s and then every minute thereafter for 35 min ($f_1, f_2, f_3, \ldots$). If the fluorescence of the final reading has not declined by >95% from the first reading, the diluted sample is reanalyzed until a satisfactory fluorescent reading is achieved. FL was prepared in a 75 mM phosphate buffer. FL working solution was routinely incubated in a water bath at 37 °C for 15 min before loading into the COBAS reagent rack. Phosphate buffer was used as a blank, and gallic acid concentrations of 800, 600, 400, 200, and 100 μM were used as standards. Catechin concentrations of 300, 200, and 100 μM were used as QC samples, and caffeic acid (125 μM) was used as the ruggedness test. The final values are calculated by using a regression equation between the gallic acid concentration and the net area under the FL decay curve. The area under curve (AUC) is calculated as

$$\mathrm{AUC} = 0.5 + f_1/f_0 + f_2/f_0 + f_3/f_0 + f_4/f_0 + \ldots + f_{34}/f_0 + 0.5(f_{35}/f_0) \quad (1)$$

where f_0 = the initial fluorescence reading at 0 min and f_i = the fluorescence reading at time i. The data were analyzed by applying eq 1 in a Microsoft Excel (Microsoft, Roselle, IL) spreadsheet to calculate the AUC. The net AUC is obtained by subtracting the AUC of the blank from that of the sample. The relative HORAC value (gallic acid equivalents, GAE) is calculated as for pure compounds (5):

$$\text{relative HORAC value} = [(\mathrm{AUC}_{\mathrm{sample}} - \mathrm{AUC}_{\mathrm{blank}})/(\mathrm{AUC}_{\mathrm{gallic\ acid}} - \mathrm{AUC}_{\mathrm{blank}})] \times (\text{molarity of gallic acid/molarity of sample}) \quad (2)$$

For mixtures, such as food samples, eq 3 is used instead:

$$\text{relative HORAC value} = [(\mathrm{AUC}_{\mathrm{sample}} - \mathrm{AUC}_{\mathrm{blank}})/(\mathrm{AUC}_{\mathrm{gallic\ acid}} - \mathrm{AUC}_{\mathrm{blank}})] \times (\text{molarity of gallic acid/concentration of sample}) \quad (3)$$

The HORAC unit is expressed as micromoles of GAE per gram for solid samples and as micromoles of GAE per liter for liquid samples.

Hydroxylation of *p*-Hydroxybenzoic Acid. *p*-Hydroxybenzoic acid (1000 mM, in water, 1.0 mL) was incubated with H_2O_2 (1.1 M, 0.5 mL) and CoF_2−PA (0.0162 M) for 30 min. The reaction mixture was analyzed by HPLC-MS. A Zorbax (Hewlett-Packard) C18 column (2.1 × 150 mm, 3 μm) was used. The mobile phase was 70% methanol with a flow rate of 0.3 mL/min, and the UV detector was set at 280 nm. The oxidized products were characterized by using a Finnigan LCQ ion trap mass spectrometer equipped with an API chamber and an ESI source. The ionization mode was negative, and the auxiliary gas and sheath gas were set to 72 and 14 units, respectively. An ionization reagent of 1.5 mM ammonium hydroxide was added at a rate of 0.05 mL/min through a tee device by using a secondary HPLC pump before the API chamber. *p*-Hydroxybenzoic acid was used as a standard for calibrating the system.

RESULTS

Metal ion induced hydroxyl radical generation reaction can be conveniently monitored by the fluorescence decay of fluorescein (FL) due to reaction 4. In the presence of preventive

$$\mathrm{FL} + \mathrm{M(II)} + \mathrm{H_2O_2} \rightarrow \text{oxidized FL (loss of fluorescence)} \quad (4)$$

$$\mathrm{M(II)} + \mathrm{L} \rightarrow \mathrm{M(II)(L)}\ \text{(inactive in reaction 4)} \quad (5)$$

antioxidant, the formation of hydroxyl radical can be inhibited because the metal is deactivated due to coordination with antioxidant, L (eq 5). The product, M(II)L, is not reactive to H_2O_2 for generating hydroxyl radicals. As such, the degree of inhibition of reaction 4 is the index of preventive antioxidant capacity, which can be quantified from the fluorescence decay curves.

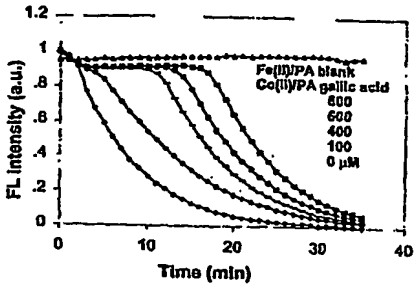

Figure 1. Fluorescent decay curve of fluorescein in the presence of gallic acid. $[FeF_2] = 230\ \mu M$, $[PA] = 460\ \mu M$, $[CoF_2] = 230\ \mu M$, $[H_2O_2] = 0.055$ M, and $[FL] = 6.20 \times 10^{-8}$ M. $T = 310$ K (37 °C). Gallic acid concentration is given in the figure.

Selection of Metals for Fenton-like Reaction. We have tested three different metal salts for Fenton reactions, Cu(I), Fe(II), and Co(II), and found that Co(II) is the most suitable metal for analytical application. Cu(I) salts are only sparely soluble, and the Fe(II) salts, albeit the most often used for Fenton reaction under acidic conditions, are too air sensitive and somehow do not cause fluorescence intensity to decay under neutral pH conditions (Figure 1). Cobalt(II) salts such as cobalt chloride and fluoride are not soluble in water either, but their complexes with 2 equiv of picolinic acid (PA) are readily soluble. Figure 1 shows the fluorescence decay curve in the presence of CoF_2/PA and FeF_2/PA. Apparently, under the physiological pH conditions, the CoF_2/PA system gives convenient fluorescence decay curves. The net area under the curve is sensitive to the concentration of antioxidants. Co(II) and H_2O_2 concentrations are selected so that the reaction finishes within 35 min, the same as the ORAC assays. Therefore, this assay can be conveniently integrated to the same instrument settings as the ORAC assay.

Evidence of Hydroxyl Radical Involvement. The involvement of HO• in the CoF_2/PA–H_2O_2 system is indirectly confirmed by using *p*-hydroxybenzoic acid as an HO• trap (*8*). The HPLC chromatogram for the reaction between *p*-hydroxybenzoic acid and CoF_2/PA–H_2O_2 is shown in Figure 5, in which the major oxidized product has been identified as 3,4-dihydroxybenzoic acid. The hydroxylation of *p*-hydroxybenzoic acid clearly indicates that HO• is involved in the mixture of *p*-hydroxybenzoic acid and CoF_2/PA–H_2O_2.

Method Validation. After the assay protocol was established, we then fully examined the validity of the assay by testing the following validity parameters.

(a) Linearity and Range. The linear relationship between the net area and the antioxidant concentration was evaluated using gallic acid at different concentrations. By integrating the areas under the fluorescent decay curve, we are able to quantify the hydroxyl radical inhibition capacity of antioxidants. The inhibition capacity is expressed as gallic acid equivalent (GAE) or HORAC value, which is quantified by the integration of the area under the curve (AUC), similar to that of ORAC assay (*9*). Figure 2 shows the nearly perfect linear relationship ($R^2 > 0.99$) between the gallic acid concentration and the AUC. Table 1 summarizes the correlation coefficient, slope, and intercept of the gallic acid standard curve. The linearity range of the assay is between 100 and 800 μM. The slopes of the linear curve fall in the range of 0.0317 ± 0.002. The intercept has a much larger variability from one run to the other. Therefore, for each HORAC assay, a standard curve should be obtained within the run and used for computation of HORAC values of samples within the same run in order to eliminate the uncertainty of the intercepts. Table 2 shows the net areas corresponding to the different concentrations of representative phenolic antioxidants and food samples. All analyzed samples demonstrate a good linear relationship between the net area and certain concentration ranges. Therefore, this assay is readily applicable to food antioxidant activity measurements.

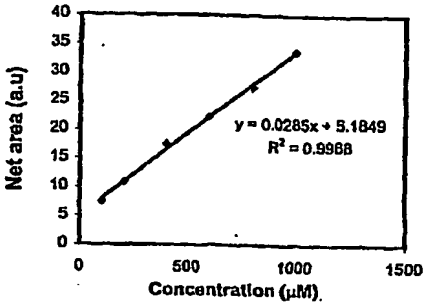

Figure 2. Linear curves of net area under the fluorescence decay curve and the gallic acid concentrations.

Table 1. Summary of Gallic Acid Calibration Curve [Y(Net Area) = $a + bX$ (μM)] Concentrations Used: 800, 400, 200, and 100 μM

run	R^2	slope (b)	intercept (a)
1	0.9885	0.0281	4.8571
2	0.9991	0.0306	4.3244
3	0.9865	0.0317	4.4690
4	0.9922	0.0320	4.7747
5	0.9990	0.0335	3.1196
6	0.9925	0.0347	3.6113
7	0.9861	0.0324	2.1751
8	0.9946	0.0310	5.550
9	0.9951	0.0330	1.2938
10	0.9971	0.0296	5.201
av	0.9931 ± 0.0048	0.0317 ± 0.002	3.94 ± 1.37

Table 2. Net Area under the Curve Corresponding to Different Compounds and Food Matrix

compound	concn (μM)	net AUC	HORAC (GAE)	R^2
rutin	100	21.1	3.82	0.9955
	80	15.91	3.61	
	60	12.68	3.83	
	40	8.31	3.77	
caffeic acid	500	26.40	1.43	0.9859
	250	16.54	1.20	
	187.5	12.46	1.30	
	125	10.40	1.51	
ferulic acid	500	23.02	1.31	0.9941
	250	12.91	1.37	
	187.5	9.40	1.33	
	125	6.85	1.46	
quercitin	250	18.38	1.59	0.9876
	125	9.90	1.72	
	100	8.50	1.84	
	75	6.28	1.81	
wild blueberry	1.3[a]	24.39	284[b]	0.9967
	0.65	10.16	237	
	0.325	4.60	215	
cranberry juice	0.2[c]	30.45	3043[d]	0.9976
	0.1	14.39	2876	
	0.05	6.584	2632	
	0.025	3.24	3236	
green tea leaf	0.66[a]	20.64	476[b]	1
	0.26	10.52	606	
	0.13	5.42	624	

[a] g/L. [b] μmol of GAE/g. [c] v/v. [d] μmol of GAE /L.

(b) Ruggedness. Day to day reproducibility was evaluated using caffeic acid. HORAC values of 125 μM caffeic acid using

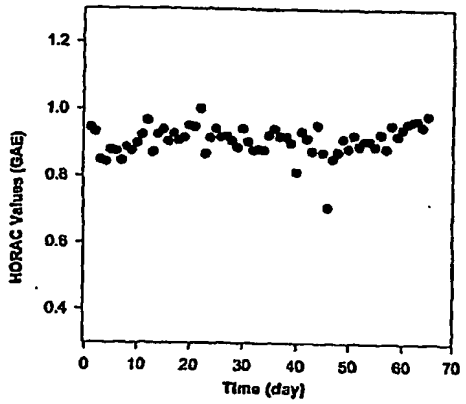

Figure 3. HORAC values of caffeic acid (125 μM) over 65 days.

Table 3. Precision and Accuracy of Quality Control (QC) Samples

catechin	QC1	QC2	QC3
norminal concn (μM)	300	200	100
run 1			
intramean (μM)	289.23	217.05	93.70
SD	18.14	11.79	2.69
%RSD	6.27	5.43	2.87
%REC	96.41	108.52	93.70
n	4	4	4
run 2			
intramean (μM)	292.05	213.22	94.71
SD	6.41	3.50	3.22
%RSD	2.19	1.64	3.40
%REC	97.3	106.61	94.71
n	4	4	4
run 3			
intramean (μM)	270.74	225.25	87.19
SD	9.96	4.70	6.84
%RSD	3.68	2.09	7.85
%REC	90.20	112.61	87.23
n	4	4	4
pooled runs			
intermean (μM)	284.01	218.51	91.87
SD	11.58	76.15	4.08
%RSD	7.82	2.81	4.44
%REC	94.67	109.25	91.87
n	12	12	12

two COBAS FARA II analyzers for 65 days are shown in Figure 3. Within the time of testing, the average HORAC value of caffeic acid is 0.90, and the percent relative standard deviation (%RSD) is 4.9%. Therefore, the assay is stable from day to day.

(c) Accuracy and Precision. Table 3 summarizes the precision and accuracy. The precision, which is expressed as the relative standard deviation (%RSD) for all QC concentrations, was within ±15%. The accuracy of the method varies from 87 to 112% within individual batches and from 92 to 109% among all of the batches. The limit of quantitation (LOQ) is 100 μM, and the limit of detection (LOD) is 50 μM GAE.

HORAC Values of Antioxidant Samples. To obtain structure and HORAC activity relationships of common phenolic compounds, we selectively examined representative phenolic compounds, and the results are shown in Table 4. For application purposes, we measured the HORAC values of common fruits and extracts as shown in Figure 4.

DISCUSSION

Conditions of Metal-Mediated Fenton Reaction. The name "Fenton reagent" refers to a mixture of hydrogen peroxide and ferrous salts, which is an effective oxidant of a large variety of organic substrates. In 1894, Fenton discovered that in the presence of a low concentration of ferrous salts and H_2O_2, tartaric acid is oxidized to dihydroxymaleic acid (*10*). In a later paper by Haber and Weiss, the authors suggested that in the decomposition of H_2O_2 catalyzed by iron salts, $HO^{\bullet}$ is formed as an active intermediate. The involvement of hydroxyl radicals as a reactive intermediate in the Fenton reagent was suggested by the fact that it is an efficient hydroxylating agent (*11*). It was found later that numerous metal ions and their complexes in their lower oxidation states [e.g., Cu(I), Ti(III), Cr(II), and Co(II)] react with H_2O_2 in a similar pattern as Fe(II), and the mixtures of these metals with H_2O_2 were thus christened "Fenton-like" reagents (*12*, *13*). Studies have demonstrated that the electron transfer reaction between a transition metal and H_2O_2 does not follow an outer-sphere electron transfer mechanism. Instead, the reaction occurs through an inner-sphere electron transfer process, in which H_2O_2 forms a complex with transition metals before electron transfer takes place (*14*). Therefore, if the transition metal is coordinatively saturated, it does not react with H_2O_2 to give reactive oxygen species. Indeed, Halliwell et al. have shown that iron chelators inhibit the Fe(II)-mediated Fenton reaction and therefore prevent the oxidative damage caused by Fe(II)/H_2O_2 (*3*). However, quantitative measurement of hydroxyl radical scavenging capacity has been a challenging task because of a lack of a controllable hydroxyl radical source. Cao et al. reported that the hydroxyl radical was generated by the mixture of H_2O_2 and Cu(II), and its scavenging capacity was quantified by the ORAC assay using

Table 4. HORAC Values of Common Natural Antioxidants

compound	HORAC (GAE)[a]	ORAC (TE)[b]
protocatechuic acid	1.04 ± 0.27	5.21 ± 0.10
gallic acid	1.00	0.90
caffeic acid	1.51 ± 0.06	4.37 ± 0.24
chlorogenic acid	1.30 ± 0.07	3.14 ± 0.19
ferulic acid	1.36 ± 0.05	3.77 ± 0.10
ECG	2.28 ± 0.19	3.6 ± 0.07
EGC	1.97 ± 0.07	2.5
catechin	2.15 ± 0.16	6.40 ± 0.23
EGCG	3.10 ± 0.41	4.94 ± 0.21
isoquercitrin	5.10 ± 0.58	4.50 ± 0.20
rutin	4.5 ± 0.2	4.28 ± 0.25
quercetin	1.79 ± 0.05	4.38 ± 0.22
kaempferol	1.71 ± 0.03	5.22 ± 0.41
genistein	2.48 ± 0.37	5.93 ± 0.45
Trolox	~0	1.0
vitamin C	~0	0.95

[a] GAE, gallic acid equivalent. [b] TE, Trolox equivalent.

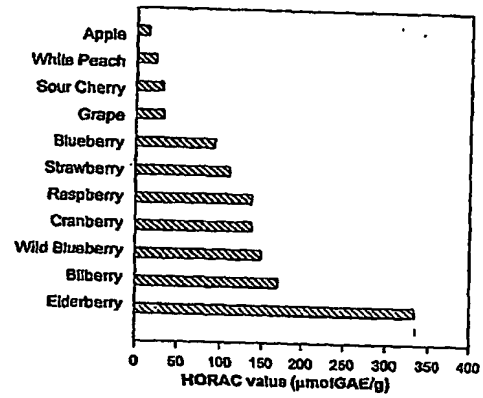

Figure 4. HORAC rankings of fruit extracts ($n = 3$).

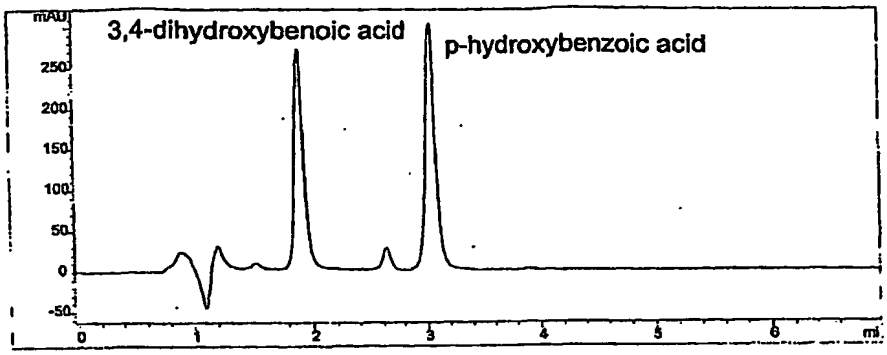

Figure 5. HPLC chromatogram of a reaction mixture of *p*-hydroxybenzoic acid and CoF_2/PA–H_2O_2. A mixture of *p*-hydroxybenzoic acid (1000 μM, in water, 1.0 mL) H_2O_2 (1.1 M, 0.5 mL), and CoF_2–PA [0.0162 M Co(II), 0.5 mL] was incubated at 37 °C for 30 min. HPLC conditions: column, Zorbax (Hewlett-Packard) C18 (2.1 × 150 mm, 3 μm); mobile phase, 70% methanol; flow rate, 0.3 mL/min; UV detector at 280 nm.

B-phycoerythrin (B-PE) as the fluorescent probe (*15*). We were unable to replicate Cao's work when fluorescein was used in place of B-PE. Although the standard redox potential of H_2O_2 (1.77 V, NHE) is much larger than that of Cu(III)/Cu(II) (0.45–1.02 V, NHE) (*16*), kinetically, the reaction may be too slow for any practical significance.

Our study also found that Fe(II) and Cu(I) compounds are prone to be oxidized in the air under neutral conditions (pH 7.4), and thus the mixture of Fe(II) or Cu(I) with H_2O_2 is not a stable source of hydroxyl radicals for quantitation. Therefore, the key elements of our study involve the selection of a metal ion stable at pH 7.4 and a means to control the oxidative reaction rate so that the decay of FL fluorescence can be completely monitored in a certain period of reaction time. After testing several metal ions, we found that Co(II) salts are air stable under physiological conditions and that the rate of hydroxyl radical formation can be controlled by the addition of PA as a chelating ligand of Co(II). At present the exact nature of the reaction between Co(II)/PA and H_2O_2 is not known and demands further investigation.

Hydroxyl Radical Prevention Capacity and Molecular Structure Relationship. After surveying the HORAC values of various phenolic compounds, we observed that the compounds that can chelate metals show significant HORAC values. Those without chelating ability have negligible HORAC values. For example, vitamin C and Trolox, the well-known radical chain-breaking antioxidants, show no hydroxyl radical prevention capability under current experimental conditions. This seems to be very puzzling at this moment. Although the lack of metal-chelating ability of vitamin C and Trolox certainly does not slow the reaction between Co(II) and H_2O_2, we still expect some protective effect due to their radical absorption capacity as reflected by their ORAC values. We suggest that they act as pro-oxidants by reduction of Co(III) to Co(II), thus initiating a catalytic cycle of hydroxyl radical generations. By doing so, they are destroyed quickly under the assay conditions, which include a large excess of hydrogen peroxide. Therefore, overall they do not show protective effects on fluorescence decay, although they may consume equal molar hydrogen peroxide. In fact, vitamin C has been used as a component of HO• radical source mediated by Fe(II).

Figure 6 represents the chemical structures of three common types of phenolics studied herein. The first group, including gallic acid, caffeic acid, ferulic acid, protocatechuic acid, and chlorogenic acid, are phenolic acids with an *ortho* diphenol

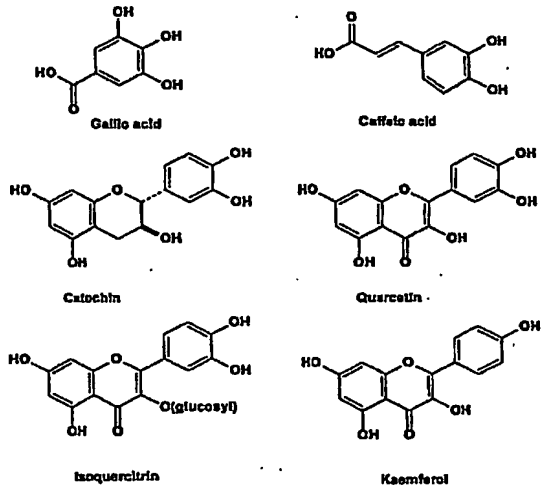

Figure 6. Chemical structures of some phenolics studied in this paper.

group. Their HORAC values fall in a narrow range of 1.0–1.51. The second group belongs to flavanol compounds including catechin, epicatechin gallate, epigallocatechin, and epigallocatechin gallate. Besides the ortho dihydroxyl groups on the B ring, they also have hydroxyl groups on the A and C rings. These compounds have higher HORAC values than the phenolic acid group (2.15–3.1 GAE). The third type is flavones with a ketone group on the 4-position of the C ring, including quercetin, genistein, kaempferol, isoquercitin, and rutin. The HORAC values of these vary largely from 1.71 (kaempferol) to 5.10 (isoquercitin). The glucose and rutinose groups have significantly positive influences on the HORAC values as the hydroxyl groups may increase the coordination ability. We have suggested that the phenolics act as metal chelators by coordination to Co(II) and therefore block the reaction sites for H_2O_2. This coordination reduces the concentration of cobalt species that are active toward H_2O_2 and thus effectively averts hydroxyl radical formation. The HORAC values primarily reflect metal coordination ability of the phenolics. The HORAC values as shown in Table 4, however, do not correlate with the number of hydroxyl groups in the phenolics and the number of potential chelating sites. This is understandable because the factor that governs the HORAC values are the stability of the complex formed between Co(II) and the phenolics (eq 6; AO =

Hydroxyl Radical Prevention Capacity Assay

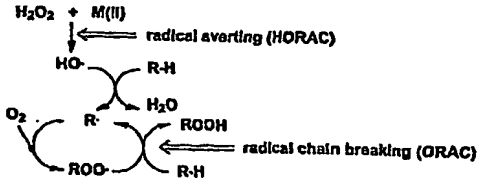

Figure 7. Relationship of HORAC and ORAC.

antioxidants). The higher equilibrium constant of eq 6 should, in principle, contribute to higher HORAC values. Further study is needed to obtain equilibrium constants of all the phenolics with Co(II) and to qualitatively establish a structure−activity relationship.

$$Co(II)(PA)_2 + AO \rightleftharpoons Co(II)(AO)(PA)_2 \qquad (6)$$

The preventive antioxidant capacity of some flavones has been well documented; for instance, metal chelation has been proposed as a mechanism for the antioxidant activities of rutin, quercetin (*17*), and butein (*18*). In addition, coordination of iron with phenolic compounds has also been employed by Yoshino and co-workers to characterize antioxidant actions of flavonoids and phenolics (*19*). Compared with the previous studies that lack a quantitation method of antioxidant capacity, the assay described herein is the first to quantitatively measure the metal-ion-chelating antioxidant capacity of food and natural products.

Comparison of HORAC and ORAC. As shown in Table 4, there is no correlation between HORAC and ORAC values. For example, genistein, quercetin, and kaempferol have very high ORAC values but their HORAC values are modest, whereas EGCG and rutin have high values for both ORAC and HORAC. In sharp contrast, the most common antioxidants, such as Trolox and vitamin C, do not show significant HORAC values, and that of melatonin is only half that of gallic acid. This phenomenon is in agreement with the fact that the HORAC and ORAC assays measure two different but equally important aspects of antioxidant properties—radical chain breaking and radical prevention. The HORAC primarily reflects metal-chelating radical prevention ability, and the ORAC reflects peroxyl radical absorption capacity. It is, therefore, expected that the samples with high HORAC values do not necessarily have high ORAC values and vice versa. Figure 7 illustrates chemically the different aspects of HORAC and ORAC assays in measuring antioxidant activity.

HORAC Values of Common Fruit Extracts. Some common fruit extracts were analyzed using the HORAC assay. The results are shown in Figure 4. Obviously, HORAC values are very sensitive to the type of fruits, and the HORAC values range broadly from 15 (apple powder) to 333 (elderberry). These values reflect the nutritional values for different fruits in hydroxyl radical prevention activity in vitro only. It would be interesting to see how the HORAC values correlate with the oxidative stress relief capacity of a biological system by these fruits in vivo.

In summary, for the first time, a novel method specific for the preventive antioxidant capacity against hydroxyl radical formation was developed and validated. As a complement to the ORAC assay that provides peroxyl radical chain-breaking capacity, the HORAC assay will be an important quantitation tool for the study of the preventive antioxidant capacity of food.

ABBREVIATIONS USED

ORAC, oxygen radical absorbance capacity; FL, fluorescein; Trolox, 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid; HORAC, hydroxyl ($HO^{\bullet}$) radical averting capacity; GAE, gallic acid equivalent; AUC, area under the curve; AO, antioxidants; PA, picolinic acid.

LITERATURE CITED

(1) Davies, K. J. A. Oxidative stress, antioxidant defenses, and damage removal, repair, and replacement systems. *IUBMB Life* 2000, *50*, 279−289.
(2) Finkel, T.; Holbrook, N. J. Oxidants, oxidative stress and the biology of aging. *Nature* 2000, *408*, 240−247.
(3) Halliwell, B.; Gutteridge, J. M. C. *Free Radicals in Biology and Medicine*, 2nd ed.; Clarendon: Oxford, U.K., 1989; pp 466−508.
(4) Huang, D.; Ou, B.; Hampsch-Woodill, M.; Flanagan, J. A.; Deemer, E. K. Development and validation of oxygen radical absorbance capacity assay for lipophilic antioxidants using randomly methylated β-cyclodextrin as the solubility enhancer. *J. Agric. Food Chem.* 2002, *50*, 1815−1821.
(5) Ou, B.; Hampsch-Woodill, M.; Prior, R. L. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *J. Agric. Food Chem.* 2001, *49*, 4619−4626.
(6) Goldstein, S.; Meyerstein, D. Comments on the mechanism of "Fenton-like" reaction. *Acc. Chem. Res.* 1999, *32*, 547−550.
(7) Halliwell, B.; Clement, M. V.; Ramalingam, J. Hydrogen peroxide. Ubiquitous in cell culture and in vivo? *IUBMB Life* 2000, *50*, 251−257.
(8) Tabatabaei, A. R.; Abbott, F. S. LC/MS analysis of hydroxylation products of salicylate as an indicator of in vivo oxidative stress. *Free Radical Biol. Med.* 1999, *26*, 1054−1058.
(9) Cao, G.; Prior, R. L. The measurement of oxygen radical absorbance capacity for biological samples. *Methods Enzymol.* 1999, *299*, 50−62.
(10) Fenton, H. J. H. *J. Chem. Soc. Trans.* 1894, *65*, 899−910.
(11) Merz, J. H.; Waters, W. A. The mechanism of oxidation of alcohols by Fenton's reagent. *Discuss. Faraday Soc.* 1947, *2*, 179−188.
(12) Samuni, A.; Meisel, D.; Czapski, G. The kinetics of the oxidation of chromium(II), titanium(III), and vanadium(IV) by hydrogen peroxide and hydroxyl radicals. *J. Chem. Soc., Dalton Trans 1* 1972, *1273*.
(13) Heckman, R. A.; Espenson, J. A. Kinetics and mechanism of oxidation of cobalt(II) macrocycles by iodine, bromide, and hydrogen peroxide. *Inorg. Chem.* 1979, *18*, 38−43.
(14) Goldstein, S.; Meyerstein, D.; Czapski, G. The Fenton Reagents. *Free Radical Biol. Med.* 1993, *15*, 435−445.
(15) Cao, G.; Alessio, H. M.; Cutler, R. Oxygen-radical absorbance capacity assay for antioxidants. *Free Radical Biol. Med.* 1993, *14*, 303−311.
(16) Cotton, F. A.; Wilkinson, G.; Murillo, C. A.; Bochmann, M. Oxygen. In *Advanced Inorganic Chemistry*, 6th ed.; Wiley: New York, 1999; pp 444−692.
(17) Afanas'ev, I. B.; Dorozhko, A. I.; Brodskii, A. V.; Kostyuk, V. A.; Potapovitch, A. I. Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation. *Biochem. Pharmacol.* 1989, *38*, 1763−1769.
(18) Cheng, Z.-J.; Kuo, S.-C.; Chan, S.-C.; Ko, F.-N.; Teng, C.-M. Antioxidant properties of butein isolated from *Dalbergia odorifera*. *Biochim. Biophys. Acta* 1998, 291−299.
(19) Yoshino, M.; Murakami, K. Interaction of iron with polyphenolic compounds: application to antioxidant characterization. *Anal. Biochem.* 1998, *257*, 40−44.

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Development and Validation of an Improved Oxygen Radical Absorbance Capacity Assay Using Fluorescein as the Fluorescent Probe

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An improved method of oxygen radical absorbance capacity (ORAC) assay has been developed and validated using fluorescein (3′,6′-dihydroxyspiro[isobenzofuran-1[3*H*],9′[9*H*]-xanthen]-3-one) as the fluorescent probe. Our results demonstrate that fluorescein (FL) is superior to B-phycoerythrin. The oxidized FL products induced by peroxyl radical were identified by LC/MS, and the reaction mechanism was determined to follow a classic hydrogen atom transfer mechanism. In addition, methodological and mechanistic comparison of $ORAC_{FL}$ with other widely used methods was discussed. It is concluded that, unlike other popular methods, the improved $ORAC_{FL}$ assay provides a direct measure of hydrophilic chain-breaking antioxidant capacity against peroxyl radical.

Keywords: *Fluorescein; ORAC; TEAC; FRAP; chain-breaking antioxidant; free radical; hydrogen atom transfer; single electron transfer*

INTRODUCTION

There is increasing interest in the use and measurement of antioxidant capacity in the food, pharmaceutical, and cosmetic industries. This interest is derived from the overwhelming evidence of importance of reactive oxygen/nitrogen species (ROS/RON) in aging and pathogenesis (*1*−*4*). Recently, Cao et al. developed a method called oxygen radical absorbance capacity (ORAC), which measures antioxidant scavenging activity against peroxyl radical induced by 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) at 37 °C (*5*, *6*). In this assay, B-phycoerythrin (B-PE), a protein isolated from *Porphyridium cruentum*, was the chosen fluorescent probe. The loss of fluorescence of B-PE is an indication of the extent of damage from its reaction with the peroxyl radical. The protective effect of an antioxidant is measured by assessing the area under the fluorescence decay curve (AUC) of the sample as compared to that of the blank in which no antioxidant is present. The ORAC assay provides a very unique and complete assessment in which the inhibition time and inhibition degree are measured as the reaction goes to completion. However, the major limitation of the $ORAC_{PE}$ assay is the use of B-PE as the probe. First, B-PE produces inconsistency from lot to lot, which results in variable reactivity to peroxyl radical (*7*). Second, B-PE is not photostable, and after exposure to excitation light for certain time, it can be photobleached. This phenomenon was observed in a 96-well plate reader where the fluorescence signal was found to decline dramatically without the addition of AAPH (unpublished results). Third, we have observed that B-PE interacts with polyphenols due to the nonspecific protein binding. These disadvantages prompted us to utilize and validate a stable fluorescent probe to replace B-PE. In this paper, we report the use of fluorescein (FL) (3′,6′-dihydroxyspiro[isobenzofuran-1[3*H*],9′[9*H*]-xanthen]-3-one) as the fluorescent probe. The FL oxidized products induced by peroxyl radical have been identified by LC/MS, and the reaction mechanism was determined to proceed as a classic hydrogen atom transfer (HAT) mechanism. Unlike other popular antioxidant activity methods, the improved $ORAC_{FL}$ assay provides a direct measure of hydrophilic chain-breaking antioxidant capacity against peroxyl radical.

MATERIALS AND METHODS

Chemicals and Apparatus. All flavonoid compounds and B-PE were purchased from Sigma (St. Louis, MO). Trolox, ascorbic acid, and disodium fluorescein were obtained from Aldrich (Milwaukee, WI). 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA (Richmond, VA). Various analyzed samples were obtained "in house". All ORAC analyses were performed on a COBAS FARA II centrifugal analyzer (Roche Diagnostic System Inc., Branchburg, NJ; excitation wavelength = 493 nm and emission filter = 515 nm).

Sample Preparation. Ascorbic acid and flavonoids were directly dissolved in acetone/water mixture (50:50, v/v) and diluted with 75 mM potassium phosphate buffer (pH 7.4) for analysis. Black tea leaves, blueberry extracts, bilberry extracts, elderberry extracts, red wine extracts; grape skin extracts and grape seed extracts were initially ground in a mechanical mill to produce a fine power. Then 0.5 g of the powders were accurately weighed, and 20 mL of acetone/water (50:50, v/v) extraction solvent was added. The mixture was shaken at 400 rpm at room temperature on an orbital shaker for 1 h. The extracts were centrifuged at 14000 rpm for 15 min, and the supernatant was ready for analysis after appropriate dilution with buffer solution. For liquid samples, a 20-mL aliquot of sample was centrifuged for 15 min, and the supernatant was

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ready for analysis after appropriate dilution. Blood plasma or serum was diluted 100−200-fold with pH 7.4 phosphate buffer before analysis. To measure the ORAC in nonprotein fraction, protein was removed using 0.5 N perchloric acid (1:1; v:v; plasma:acid), the samples were then centrifuged at 14000*g* for 10 min at 4 °C, and the supernatants were removed as the serum nonprotein fractions and appropriately diluted with pH 7.4 phosphate buffer before analysis.

Experimental Conditions. *ORAC Assay.* The COBAS FARA II was programmed to use a two-reagent system. The reaction mode pipetted and transferred the sample (20 μL), phosphate buffer (5 μL, 75 mM, pH 7.4), and main reagent (365 μL FL, 48 nM) into the main reagent wells of their respective cuvette rotor positions. With spinning of the rotor, the reagents were mixed and incubated for 30 s before recording the initial fluorescence (f_0). Fluorescence readings were taken at 0.5 s and then every minute thereafter (f_1, f_2, f_3, ...) for a duration of 30 min. To determine the maximum voltage for the photomultiplier tube, the AAPH reagent was replaced with buffer, and the analysis was run for 10 min. FL and AAPH were prepared with 75 mM phosphate buffer at pH 7.4. FL working solution was preincubated at 37 °C for 15 min before loading into the COBAS reagent rack. The 75 mM phosphate buffer was used as a blank, and 12.5, 25, 50, and 100 μM Trolox were used as standards. A sample of 40 μM Trolox was used as quality control (QC). Samples and Trolox calibration solutions were always analyzed in duplicate in a "forward-then-reverse" order as follows: blank, 12.5 μM Trolox, 25 μM Trolox, 50 μM Trolox, 100 μM Trolox, QC, sample 1 ... sample 1, QC, 100 μM Trolox, 50 μM Trolox, 25 μM Trolox, 12.5 μM Trolox, blank. This arrangement can correct possible errors due to the signal drifting associated with the different positions of the same sample. The final ORAC values were calculated by using a regression equation between the Trolox concentration and the net area under the FL decay curve and were expressed as Trolox equivalents as micromole per liter or per gram. The area under curve (AUC) was calculated as

$$\mathrm{AUC} = 1 + f_1/f_0 + f_2/f_0 + f_3/f_0 + f_4/f_0 + \ldots + f_{34}/f_0 + f_{35}/f_0 \quad (1)$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time i.

The data were analyzed by applying eq 1 in a Microsoft Excel (Microsoft, Redmond, WA) spreadsheet to calculate the AUC. The net AUC was obtained by subtracting the AUC of the blank from that of the sample. The relative ORAC value (Trolox equivalents) was calculated as

$$\text{relative ORAC value} = [(\mathrm{AUC_{Sample}} - \mathrm{AUC_{Blank}})/(\mathrm{AUC_{Trolox}} - \mathrm{AUC_{Blank}})] \times (\text{molarity of Trolox/molarity of sample}) \quad (2)$$

Characterization of FL Oxidized Products. FL (4.8×10^{-7} M) was incubated at 37 °C for 20 min with AAPH (1.28×10^{-2} M) at 75 mM potassium phosphate buffer (pH 7.4), and the reaction mixture was analyzed by LC/MS. Chromatographic analyses were performed on an HP 1100 series (Hewlett-Packard, Palo Alto, CA) HPLC equipped with an autosampler/injector, binary HPLC pump, column heater, diode array detector, fluorescence detector, and HP ChemStation for data collection and manipulation. Reverse-phase separation was performed on a Zorbax (Hewlett-Packard, Palo Alto, CA) C18 column (2.1 × 150 mm, 3 μm) at 37 °C. UV detection was recorded at 278 nm, and for fluorescence detection, the excitation wavelength was 491 nm and the emission wavelength was 515 nm. The binary mobile phase consisted of (A) water−acetonitrile−acetic acid (89:9:2) and (B) water−acetonitrile (20:80). The separation was performed using a linear gradient from 0% to 30% B in 30 min. The structural information was obtained using a Finnigan LCQ ion trap mass spectrometer (Thermoquest, San Jose, CA) equipped with an API chamber and an ESI source. The ionization mode was negative mode; Aux gas and Sheath gas were set to 90 and 23 units, respectively. An ionization reagent of 1.5 mM ammonium hydroxide was added at a rate of 0.05 mL/min through a Tee device by using a secondary HPLC pump before the API chamber. Fluorescein disodium was used as a standard for calibrating the system.

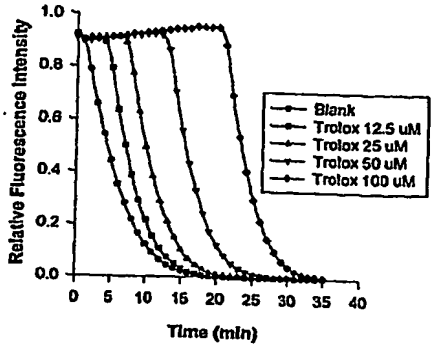

Figure 1. Trolox concentration effect on FL fluorescence decay curve.

Table 1. Summary of Trolox Calibration Curve[a]

run	R^2	slope (b)	intercept (a)
1	0.9994	2.5368	−2.174
2	0.9993	2.7390	−4.690
3	0.9981	2.6947	−5.109
4	0.9973	2.5291	−3.846
5	0.9928	2.2331	1.361
6	0.9978	2.8868	−3.788
7	0.9981	2.6288	−3.012
8	0.9987	2.5297	−2.589
average	0.9977	2.5846	−2.861
accepted criteria	≥0.9900	na	na

[a] [Y (μM) = $a + bX$ (net area)]. na, not applicable.

RESULTS

Specificity. The purpose was to demonstrate whether the improved method is specific for antioxidants. This objective can be confirmed by obtaining positive results from a sample containing antioxidants and negative results from a same sample whose antioxidants have been destroyed. The following were chosen for specificity determination: 100 μM gallic acid, 3% blueberry juice, and whole serum. After preincubation with 1.28×10^{-2} M AAPH and Fenton reagent ($H_2O_2 + Fe^{2+}$) at 37 °C for 2 h, all three samples were found to have no scavenging activities while showing negative ORAC values. Therefore, the $\mathrm{ORAC_{FL}}$ assay is specific for antioxidants.

Linearity. The linear relationship between net area and antioxidant concentration was evaluated using Trolox, black tea leaves, blueberry extracts, and grape skin extracts at different concentrations. Figure 1 illustrates the FL fluorescence decay curves in the presence of Trolox and AAPH. Table 1 summarizes the correlation coefficient, slope, and intercept of the Trolox standard curve. Table 2 shows the net areas corresponding to the different concentrations of black tea leaves, blueberry extracts, and grape skin extracts and the calculated ORAC values. All analyzed samples in the various forms demonstrate a good linear relationship between net area and concentration. Trolox was used as a calibration standard. The limit of quantitation and the limit of detection are 12.5 and 5 μM Trolox equivalents, respectively. An acceptable correlation of coefficient (r^2) was ≥0.99.

Table 2. Net Area Corresponding to Different Concentrations of Extracts from Tea, Blueberry, and Grape Skins

natural products	concn(mg/L)	net area	$ORAC_{FL}$[a]
black tea leaves	8	5.92	1586
	16	10.81	1566
	32	21.51	1629
blueberry extracts	5	5.73	2441
	10	11.32	2635
	20	22.98	2792
grape skin extracts	1.2	8.34	15675
	2.4	15.63	15521
	4.8	29.89	14714

[a] ORAC values are expressed as Trolox equivalents per gram on dry basis. The relative standard deviation (RSD) for average value of each sample was less than 15%.

Table 3. Precision and Accuracy of Quality Control (QC) Samples[a]

	QC1	QC2	QC3
nominal Trolox concn (μM)	20.00	40.00	75.00
run 1			
intra-mean (μM)	18.21	41.81	74.79
SD	1.26	3.51	5.49
% RSD	6.90	8.40	7.34
% REC	91.05	100.05	99.72
n	4	4	4
run 2			
intra-mean (μM)	21.33	42.79	76.18
SD	1.58	3.92	6.12
% RSD	7.41	9.16	8.03
% REC	106.65	107.03	101.57
n	4	4	4
run 3			
intra-mean (μM)	21.45	41.35	76.21
SD	1.37	3.21	5.19
% RSD	6.39	7.76	6.81
% REC	107.25	103.35	101.61
n	4	4	4
pooled runs			
inter-mean (μM)	20.33	41.98	75.72
SD	1.59	0.74	0.81
% RSD	7.82	1.76	1.16
% REC	101.65	104.95	100.96
n	12	12	12

[a] % RSD, relative standard deviation. % REC, relative recovery.

Precision and Accuracy. Table 3 summarizes the precision and accuracy of the $ORAC_{FL}$ assay. The precision, which is expressed as relative standard deviation (% RSD) for all quality control samples, was within ±15%. The accuracy of the method varies from 91 to 107% within individual batches and from 101 to 105% between all the batches.

Ruggedness. The reproducibility of $ORAC_{FL}$ was evaluated by a ruggedness study, which was performed by analyzing 20 μM gallic acid using two COBAS FARA II analyzers day to day. Results are shown in Figure 2.

Comparison of FL with B-PE. We performed analyses for grape seed extracts (GSE) in the absence of AAPH by using both B-PE and FL. Figure 3a shows a strong protein binding between B-PE and GSE that occurred instantly. The degree of binding appears to be inversely proportional to GSE concentration. No interaction was observed from FL and GSE matrix within 35 min (Figure 3b). Figures 4 and 5 represent the FL and B-PE fluorescence decay curves, respectively, and as shown, B-PE fluorescence declined more rapidly than did FL fluorescence. Various samples were analyzed, and the results are summarized in Tables 4–6. The FL yields a consistently higher ORAC value as compared to B-PE (~1.6–3.5-fold).

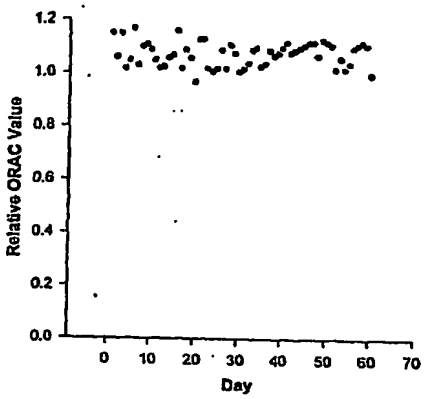

Figure 2. Ruggedness of ORAC method determined by 20 μM gallic acid. The relative ORAC values were obtained from two COBAS FARA II analyzers over several different days.

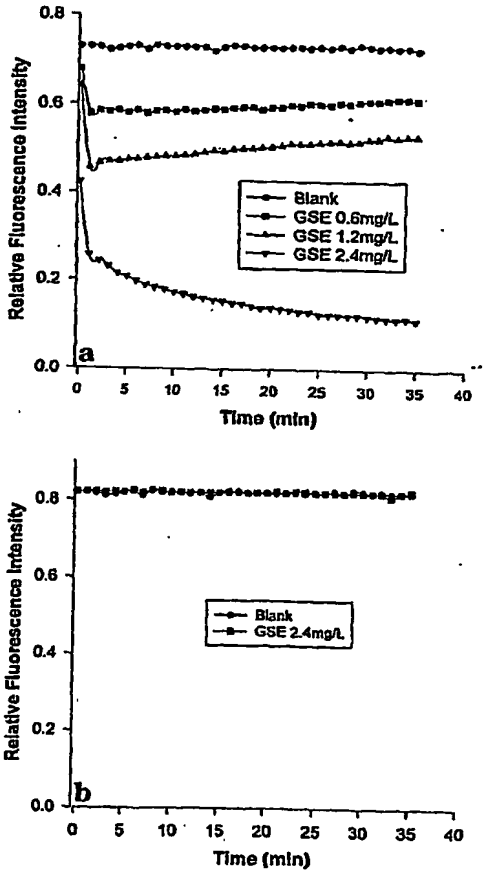

Figure 3. (a) Relative fluorescence versus time (minutes) of reaction: blank and grape seed extracts (GSE) at various concentrations using B-PE as the fluorescent probe. (b) Blank and GSE at 2.4 mg/L using FL as the fluorescent probe.

Mechanistic Studies. The mechanisms for peroxidation of FL can be elucidated based on FL oxidized products. As shown in Figure 6, FL was oxidized into three minor fluorescent products (FL1, FL2, FL3) and one major nonfluorescent product (FL4) with a maximum absorption at 278 nm. Figure 7 illustrates the proposed structures of FL oxidized products and the

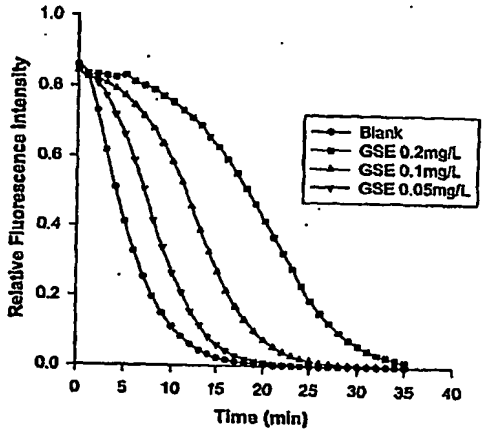

Figure 4. FL fluorescence decay curve induced by AAPH in the presence of grape seed extract (GSE) at different concentrations.

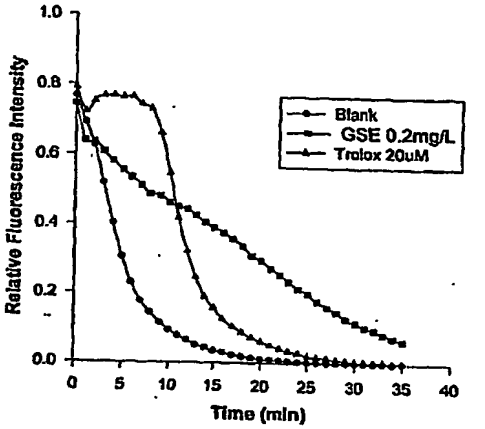

Figure 5. B-PE fluorescence decay curve induced by AAPH in the presence of 4 mg/mL grape seed extract (GSE).

Table 4. Relative ORAC Values of Pure Chemicals with Antioxidant Activity[a]

compounds	$ORAC_{FL}$	$ORAC_{PE}$	ratio
caffeic acid	4.37 ± 0.24	1.40 ± 0.09	3.12
chlorogenic acid	3.14 ± 0.19	1.90 ± 0.12	1.65
quercetrin	6.47 ± 0.29	2.70 ± 0.18	2.39
genistein	5.93 ± 0.45	2.3 ± 0.16	2.58
glutathione	0.62 ± 0.02	0.32 ± 0.01	1.94
rutin	6.01 ± 0.25	1.95 ± 0.21	3.08
quercetin	7.28 ± 0.22	2.07 ± 0.05	3.52
catechin	6.76 ± 0.22	2.57 ± 0.18	2.63
vitamin C	0.95 ± 0.02	0.43 ± 0.03	2.21

[a] ORAC values are expressed as relative Trolox equivalent calculated based on eq 2 ($n > 3$).

oxidation scheme. The chromatographic and mass spectroscopic data for the oxidized products are summarized in Table 7.

DISCUSSION

The original $ORAC_{PE}$ was based largely on Glazer's work (*8*) in which B-PE was utilized as the fluorescent probe. The reason for choosing B-PE as the fluorescent probe is due to B-PE's distinct excitation and emission wavelengths, high fluorescence yield, sensitivity to ROS, and water solubility (*9*). Later, the developers of the

Table 5. $ORAC_{FL}$ and $ORAC_{PE}$ Values for Biological Fluids and Beverages[a]

sample	$ORAC_{FL}$	$ORAC_{PE}$	$ORAC_{FL}$/ $ORAC_{PE}$
urine	1542 ± 178	926 ± 133	1.67
whole serum	7780 ± 467	3383 ± 278	2.30
serum (protein free)	347 ± 5.63	186 ± 9.11	1.87
blueberry juice	23748 ± 1555	7511 ± 683	3.16
bilberry juice	34659 ± 2069	12507 ± 893	2.77
grape juice	31441 ± 1821	12124 ± 912	2.59
raspberry juice	54034 ± 2863	23056 ± 1800	2.34
black tea	17267 ± 441	8714 ± 213	1.89

[a] ORAC values are expressed as micromol of Trolox equivalent per liter ($n > 3$).

Table 6. $ORAC_{FL}$ and $ORAC_{PE}$ of Various Natural Product Extracts[a]

sample	$ORAC_{FL}$	$ORAC_{PE}$	$ORAC_{FL}$/ $ORAC_{PE}$
bilberry	2646 ± 190	1283 ± 144	2.06
elderberry	2221 ± 164	1174 ± 182	1.89
red wine extract	6942 ± 669	2194 ± 105	3.16
grape seeds extract A	11889 ± 234	3516 ± 135	3.38
grape seeds extract B	11681 ± 923	2989 ± 368	1.89

[a] $ORAC_{FL}$ and $ORAC_{PE}$ values are expressed as micromol of Trolox equivalents per gram ($n > 3$).

Table 7. Ion Trap Mass Data for FL and Its Major Oxidized Products[a]

compound	RT (min)	λ_{max}	$[M-1]^-$	MS^2	MS^3	MS^4
fluorescein	30.4	493	331.1	287.2	259.3	
FL1	24.4	493	661.0	617.1	269.2 573.3	243.3 545.2
FL2	28.8	493	375.0	331.1	589.0 287.2	 259.2
FL3	26.4	345	349.0	305.0	261.1	233.1
FL4	3.1	278	221.3		204.2	187.1

[a] See Figure 6 for the HPLC separation of these products and Figure 7 for the proposed structures of FL oxidized products. FL4 is an unidentified product.

$ORAC_{PE}$ reported that different PEs, such as B-PE and R-PE, were found to possess different fluorescence intensity and reactivity to peroxyl radical; these differences even existed in the same PE with different lots. Hence, they suggested that PE of a single lot number be used for a planned project (*7*). Since the commercial available B-PE is only approximately 30% pure, the inconsistency of PE from lot to lot is very likely attributed to the isolation process from *P. cruentum*. Initially, we adapted the $ORAC_{PE}$ method to measure antioxidant activity in our laboratory. In addition to the inherent variability, we found that B-PE interacted with polyphenols (Figure 3a), a major class of antioxidants from natural products. The interaction between B-PE and polyphenols is caused by nonspecific protein binding. Complexing of polyphenols with protein has been known and studied for decades (*10*–*12*). The most important mechanism seems to involve hydrophobic interactions and also hydrogen bonding (*13*, *14*). The nonspecific protein binding causes falsely low ORAC values as demonstrated in Tables 4–6 where $ORAC_{PE}$ values are consistently lower than those of $ORAC_{FL}$. It is noted that the ratio between two values varies from 1.5 to 3.1, indicating that different compounds have different affinities to PE. Another disadvantage of B-PE is photoinstability. The $ORAC_{PE}$ method has been criticized for the lack of accessibility because of the rare availability of FARA COBAS II analyzer. We attempted

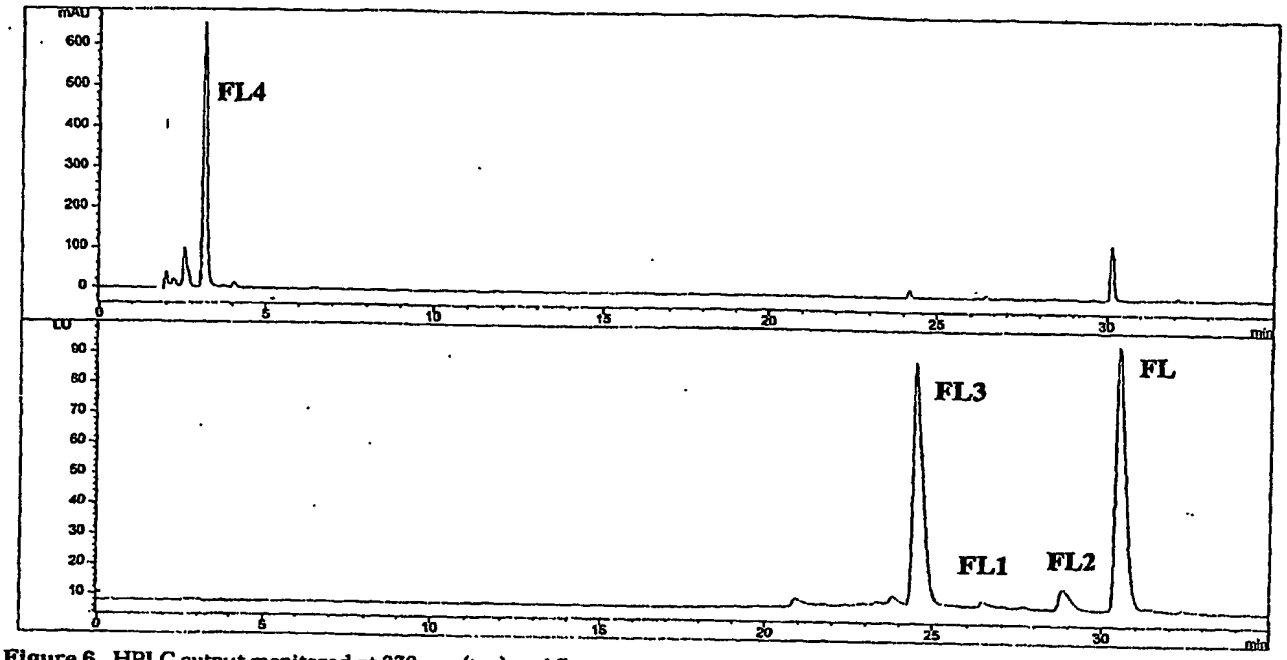

Figure 6. HPLC output monitored at 278 nm (top) and fluorescence at 493 nm excitation and 515 emission of fluorescein (bottom) and its oxidized products in the presence of AAPH.

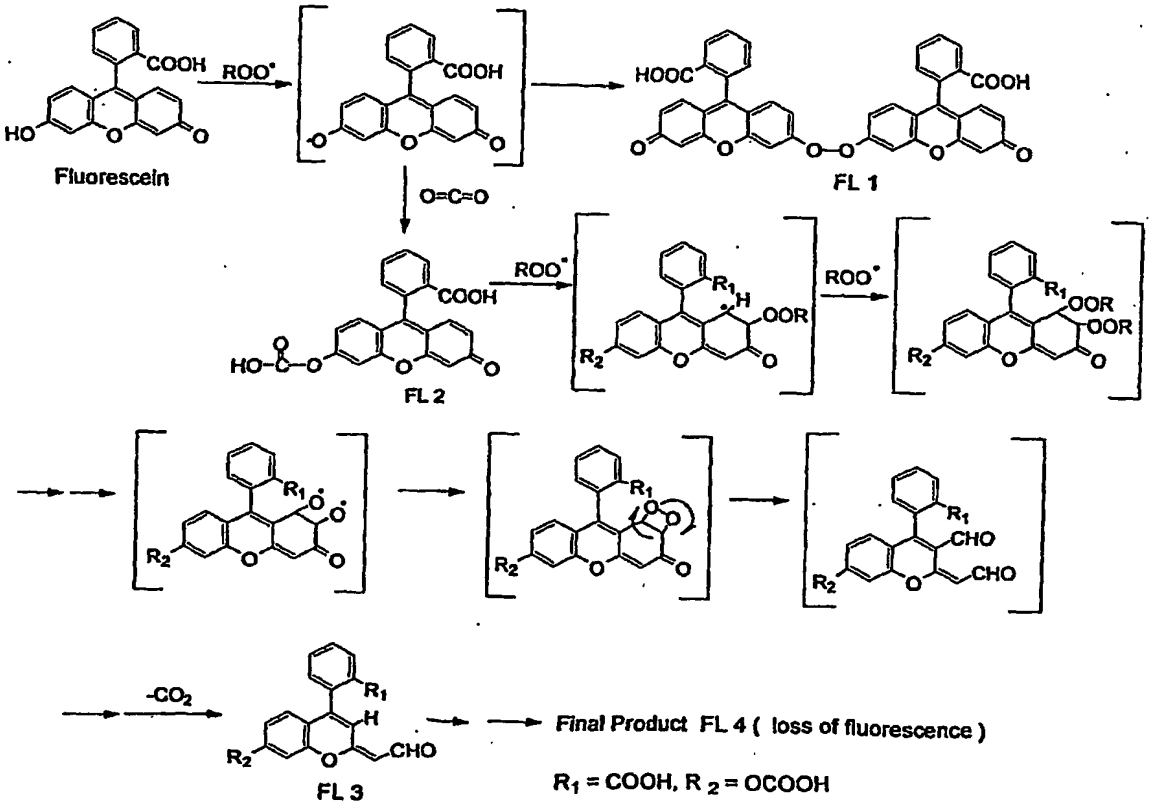

Figure 7. Proposed FL oxidation pathway in the presence of AAPH.

to adapt the $ORAC_{PE}$ method to a 96-well plate reader. Unfortunately, the fluorescence of B-PE was found to drop dramatically in a short period of time in the absence of AAPH. Moreover, being a protein isolated from *P. cruentum*, B-PE is costly. In general, 75% of the cost of $ORAC_{PE}$ is for B-PE. Clearly, in terms of the

criteria for method validation and cost-effectiveness, B-PE is less than ideal as a fluorescent probe. In contrast to PE, FL and its derivatives are the most used fluorescent probes for labeling and sensing biomolecules (*15*). Recently, Nagano et al. successfully utilized the FL based fluorescent probes to detect $^1O_2$ and NO• in vivo (*16, 17*). FL ($pK_a = 6.4$) is a synthetic compound with high quantum yield of fluorescence at pH > 7.0 ($\phi = 0.78$) and long wavelengths (492/515 nm, excitation/emission). As compared to PE, FL is extremely inexpensive. Importantly, FL does not interact with other compounds as shown in Figure 3b. Moreover, FL is very stable in a 96-well plate reader without photobleaching; this advantage makes the $ORAC_{FL}$ method more accessible to other researchers (in preparation). It is necessary to point out that the fluorescence intensity of FL is pH sensitive. When pH drops below 7, its intensity decreases greatly. However, the $ORAC_{FL}$ assay is very sensitive; samples always need to be diluted greatly with 75 mM phosphate buffer at pH 7.4 before analysis. Considering the extreme situation in which pure acetic acid is analyzed, the pH only dropped slightly from 7.4 to 7.35 based on our experimental data and theoretical calculation. Therefore, the pH sensitivity of FL does not affect the $ORAC_{FL}$ assay.

Although the FDA still has no regulations concerning standardization in the nutraceutical and food supplement industry, there are good scientific and business reasons for validating assay procedures even in the absence of regulatory or compendia requirements. In the present study, the $ORAC_{FL}$ procedure has been validated through specificity, linearity, precision and accuracy, and ruggedness. The results from validation experiments clearly demonstrate that the $ORAC_{FL}$ assay is specific for antioxidants and is sensitive, precise, and robust within accepted criteria.

Elucidation of oxidation mechanisms involved in the $ORAC_{FL}$ assay is an important part of method validation. In general, the antioxidant reactions involve multiple steps including the initiation, propagation, branching, and termination of free radicals. This whole process is termed a chain reaction. The antioxidants therefore fall into two mechanistic groups: those which inhibit or retard the formation of free radicals from their unstable precursors (initiation) are called the "preventive" antioxidants, and those which interrupt the radical chain reaction (propagation and branching) are the "chain-breaking" antioxidants. The chain-breaking antioxidants are the most studied antioxidants, and the mechanism follows the HAT mechanism. The driving force for HAT is the formation of a delocalized stable radical that does not continue the chain reaction or continues it with only a low efficiency. Specifically, a chain-breaking antioxidant (AH) donates its labile hydrogen atom to ROO• much more rapidly than ROO• reacts with substrate. The radical A• is stable and is not able to continue the autoxidation of the chain. The HAT mechanism has been extensive studied and has been widely accepted as the predominate mechanism for autoxidation initiated by oxygen radicals (*18, 19*). B-PE is multi-subunit protein with the structure of $(\alpha\beta)_6\gamma$ (*8*); it is very difficult to determine the reaction mechanisms when B-PE is used as the probe. While FL is a small organic molecule with simple structural skeleton, the oxidation mechanisms can be elucidated based on its oxidized products characterized by LC/MS. As illustrated in Figure 7, the first step of FL oxidation involves one hydrogen of the phenol group being abstracted by a peroxyl radical, forming a stable FL phenoxyl radical (FLO•) that readily undergoes dimerization to form a dimeric FL1 with *m/z* 661. Alternatively, FLO• can attack trace amounts of CO_2 in the buffer solution to yield FL2 with *m/z* 375.0. Besides hydrogen abstraction, ROO• can also add the reactive conjugated C—C double bond to form a stable delocalized radical that further reacts with ROO• to form the endoperoxide intermediate, followed by decomposition to yield FL3 with *m/z* 349.0. FL4, a major oxidative product with *m/z* 221.3, appears to be derived from FL3 due to the further oxidation. FL4 does not possess fluorescent emission at 495/515 nm. It is clear that the oxidative mechanism for the $ORAC_{FL}$ assay follows the HAT mechanism. Therefore, the $ORAC_{FL}$ assay directly measures the antioxidant activity of chain-breaking antioxidants against peroxyl radical.

Besides the $ORAC_{FL}$ assay, a number of other methods for measuring antioxidant activity have been reported over recent years. Among them, FRAP (ferric reducing/antioxidant power) (*20*) and TEAC (Trolox equivalent antioxidant capacity) (*21*) have gained popularity because they are simple and speedy. For example, TEAC and FRAP fix the reaction time at 4 and 6 min, respectively. Although several review articles on the comparison of different methods have been published (*22, 23*), little attention has been paid to basic chemical principles involved in these methods. The lack of mechanistic understanding of antioxidant will bring confusion to this important field. Therefore, we compare $ORAC_{FL}$ with other methods from a mechanistic point of view. FRAP and TEAC are the single electron-transfer mechanism instead of HAT mechanism (*20, 24*). As a result, neither TEAC nor FRAP actually measures chain-breaking antioxidant activity or preventive antioxidant activity. Specifically, the FRAP assay depends on the reduction of a ferric tripyridyltriazine [Fe(III)—$(TPTZ)_2$] complex to the ferrous tripyridyltriazine [Fe(II)—$(TPTZ)_2$] by an antioxidant at the nonphysiological condition with low pH of 3.6. On the other hand, if the FRAP assay is used to assess in vivo antioxidant status, Fe(II) can interact with H_2O_2 to produce HO•, the most harmful ROS. The original TEAC assay was based on the activation of metmyoglobin with hydrogen peroxide in the presence of ABTS to produce the radical cation. This has been criticized because the faster reacting antioxidants might also contribute to the reduction of the ferryl myoglobin radical and the added hydrogen peroxide could oxidize antioxidants before the measurement. In the improved TEAC, $ABTS^{+\bullet}$ is pregenerated by potassium persulfate (*25*). However, very similar to FRAP, any trace ions contained in biological fluids and natural products can inevitably reduce $ABTS^{+\bullet}$ to ABTS, causing falsely higher results. The standard redox potential of Fe(II)/Fe(III) is 0.77 V, and that of ABTS/$ABTS^{+\bullet}$ is 0.68 V (*26*). It must be pointed out that no oxygen radical is involved in either FRAP or TEAC ($ABTS^{+\bullet}$ is not a ROS). Therefore, FRAP/TEAC results do not necessarily reflect antioxidant activities. This conclusion can be further confirmed by the fact that FRAP does not measure the thiol antioxidants, such as glutathione. In addition, FRAP and TEAC presumably rely on the hypothesis that the redox reactions proceed so fast that all reactions are complete within a short

period of time; in fact, this is not always true. For example, Pulido and co-workers recently examined the FRAP assay of dietary polyphenols in water and methanol (*27*). The UV−vis absorption of $Fe(II)(TPTZ)_2$ was slowly increasing even after several hours of reaction time. The polyphenols with such behaviors include caffeic acid, tannic acid, ferulic acid, and quercetin. Clearly, FRAP and TEAC only partially measure the reducing capability based upon Fe(III) and $ABTS^{+\bullet}$, respectively, which is not relevant to antioxidant activity mechanistically and physiologically.

In summary, an improved ORAC assay ($ORAC_{FL}$) using FL as the fluorescent probe has been developed and validated. The validation results demonstrate that the $ORAC_{FL}$ method is robust. As compared to B-PE, FL does not interact with antioxidant samples. Meanwhile, FL shows an excellent photostability so that the $ORAC_{FL}$ assay can be transferred to a 96-well plate reader. Furthermore, the use of FL substantially reduces the cost of experiment. Therefore we suggest that FL be considered as a standard to evaluate chain-breaking antioxidant activity. On the basis of the FL oxidized products, the mechanism of FL oxidation induced by peroxyl radical is determined to follow the HAT mechanism. In contrast, the popular FRAP and TEAC follow a single electron-transfer mechanism. Hence, only the $ORAC_{FL}$ assay directly estimates the chain-breaking antioxidant activity, while the FRAP and TEAC assays actually measure the specific oxidant-reducing power not equivalent to antioxidant activity. However the $ORAC_{FL}$ assay cannot be considered a "total antioxidant activity assay" since the assay is performed in aqueous solution. Therefore, the $ORAC_{FL}$ assay primarily measures hydrophilic antioxidant activity against peroxyl radical. In fact, it is impossible to measure total antioxidant activity using only a single assay. To elucidate a full profile of antioxidant activity against various ROS/RNS, such as $O_2^{-\bullet}$, $HO^{\bullet}$, and $NO^{\bullet}$, the development of different methods specific for each ROS/RNS is needed.

ABBREVIATIONS USED

FL, fluorescein; B-PE, B-phycoerythrin; Trolox, 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid; AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; ORAC, oxygen radical absorbance capacity; GSE, grape seed extracts; HAT, hydrogen atom transfer; TEAC, Trolox equivalent antioxidant capacity; FRAP, ferric reducing antioxidant power;

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LITERATURE CITED

(1) Halliwell, B.; Aruoma, O. I. DNA damage by oxygen derived species. Its mechanism and measurement in mammalian systems. *FEBS Lett.* **1991**, *281*, 9−19.

(2) Wink, D. A.; Kasprzak, K. S.; Maragos, C. M.; Elespuru, R. K.; Misra, M.; Dunams, T. M.; Cebula, T. A.; Koch, W. H.; Andrew, A. W.; Allen, J. S.; Keefer, L. K. DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. *Science* **1991**, *254*, 1001−1003.

(3) Nguyen, T.; Brunson, D.; Crespi, C. L.; Penman, B. W.; Wishnok, J. S.; Tannenbaum, S. R. DNA damage and mutation in human cells exposed to nitric oxide in vitro. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 3030−3034.

(4) Stadtman, E. R. Protein modification in oxidative stress. In *Oxidative Processes and Antioxidants*; Paoletti, R., Sainuelsson, B., Carapano, A. L., Poli, A., Rinetti, M., Eds.; Raven Press: New York, 1994; pp 117−134.

(5) Cao, G.; Alessio, H. M.; Culter, R. Oxygen-radical absorbance capacity assay for antioxidants. *Free Radical Biol. Med.* **1993**, *14*, 303−311.

(6) Cao, G.; Verdon, C. P.; Wu, A. H. B.; Wang, H.; Prior, R. L. Automated assay of oxygen radical absorbance capacity with the COBAS FARA II. *Clin. Chem.* **1995**, *41*, 1738−1744.

(7) Cao, G.; Prior, R. L. The measurement of oxygen radical absorbance capacity in biological samples. *Methods Enzymol.* **1999**, *299*, 50−62.

(8) Glazer, A. N. Fluorescence-based assay for reactive oxygen species: A protective role for creatinine. *FASEB J.* **1988**, *2*, 2487−2491.

(9) Glazer, A. N. Phycoerythrin fluorescence-based assay for reactive oxygen species. *Methods Enzymol.* **1990**, *186*, 161−168.

(10) Haslam, E. Polyphenol-protein interactions. *Biochem. J.* **1974**, *139*, 285−288.

(11) Haslam, E. The association of proteins with polyphenols. *J. Chem. Soc., Chem. Commun.* **1981**, 309−311.

(12) Freitas, V. D.; Nateus, N. Structure features of procyanidin interactions with salivary protein *J. Agric. Food. Chem.* **2001**, *49*, 940−945.

(13) Murray, N. J.; Williamson, M. P.; Lilley, T. H.; Haslam, E. Study of the interaction between salivary proline-rich proteins and polyphenol by ^1H NMR spectroscopy. *Eur. J. Biochem.* **1994**, *219*, 923−935.

(14) Siebert, K. J.; Troukhanova, N. V.; Lynn, P. Y. Nature of polyphenol-protein ineractions. *J. Agric. Food Chem.* **1996**, *44*, 80−85.

(15) Srutkowska, S.; Caspi, R.; Gabig, M.; Wegrzyn, G. Detection of DNA replication intermediates after two-dimensional agarose gel electrophoresis using a fluorescein-labeled probe. *Anal. Biochem.* **1999**, *269*, 221−222.

(16) Tanaka, K.; Miura, T.; Umezawa, N.; Urano, Y.; Kikuchi, K.; Higuchi, T.; Nagano, T. Rational design of fluorescein-based fluorescence probes. Mechanism-based design of a maximum fluorescence probe for singlet oxygen. *J. Am. Chem. Soc.* **2001**, *123*, 2530−2536.

(17) Kojima, H.; Urano, Y.; Kikuchi, K.; Higuchi, T.; Hirata, Y.; Nagano, T. Fluorescent indicators for imaging nitric oxide production. *Angew. Chem., Int. Ed.* **1999**, *38*, 3209−3212.

(18) Wright, J. S.; Carpenter, D. J.; Mckay, D. J.; Ingold, K. U. Theoretical calculation of substituent effects on the O−H bond strength of phenolic antioxidants related to vitamin E. *J. Am. Chem. Soc.* **1997**, *119*, 4245−4252.

(19) Vinqvist, M. R.; Barclay, L. R. On the antioxidant mechanism of curcumin: classic methods are needed to determine antioxidant mechanism and activity. *Org. Lett.* **2000**, *2*, 2841−2843.

(20) Benzie, I. F.; Strain, J. J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal. Biochem.* **1996**, *239*, 70−76.

(21) Miller, N. J.; Rice-Evans, C.; Davies, M. J.; Gopinathan, V.; Milner, A. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clin. Sci.* **1993**, *84*, 407−412.

(22) Cao, G.; Prior, R. L. Comparison of the analytical methods for assessing the total antioxidant capacity of human serum. *Clin. Chem.* **1998**, *44*, 1309−1315.

(23) Rice-Evans, C. Measurement of total antioxidant activity as a marker of antioxidant status *in vivo*: procedures and limitations. *Free Radical Res.* **2000**, *33*, 559−566.

(24) Wolfenden, R. S.; Willson, R. L. Radical cation as reference chromogens in kinetic studies of one-electron-transfer reaction: plus radiolysis studies of 2,2′-azinobis-(3-ethylbenzthiaoline-6-sulphonate). *J. Chem. Soc., Perkin Trans. 2* **1982**, 805−812.

(25) Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biol. Med.* **1999**, *26*, 1231−1237.

(26) Scott, S. L.; Chen, W.; Bakac, A.; Espenson, J. Spectroscopic parameters, electrode potentials, acid ionization constants, and electron exchange rates of the 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonate) radicals and ions. *J. Phys. Chem.* **1993**, *97*, 6710−6714.

(27) Pulido, R.; Bravo, L.; Saura-Calixo, F. Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. *J. Agric. Food Chem.* **2000**, *48*, 3396−3402.

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